

The Journal of Laboratory and Clinical Medicine

CARL V. MOORE, M.D., Editor
606 South Kingshighway
St. Louis 10, Mo.

ASSOCIATE EDITORS

Pharmacology

DENNIS E. JACKSON, M.D.
University of Cincinnati, Cincinnati

Clinical Pathology

W. C. MACCARTY, M.D.
Mayo Clinic, Rochester, Minn.

T. B. MAGATH, M.D.
Mayo Clinic, Rochester, Minn.

Clinical Chemistry

VICTOR C. MYERS, Ph.D.
Western Reserve University, Cleveland

EDWARD MUNTWYLER, Ph.D.
Long Island College of Medicine, Brooklyn

Internal Medicine

GEORGE HERRMANN, M.D.
University of Texas, Galveston

Metabolism

CLIFFORD J. BARBORKA, M.D.
Northwestern University, Chicago

Tuberculosis

GERALD B. WEBB, M.D.
Colorado Springs, Colo.

Experimental Medicine

RUSSELL L. HADEN, M.D.
Cleveland Clinic, Cleveland

Immunology

JOHN A. KOLMER, M.D.
Temple University, Philadelphia

Bacteriology

M. H. SOULE, Sc.D.
University of Michigan, Ann Arbor

Pathology

PAUL R. CANNON, M.D.
University of Chicago, Chicago

Medical Illustration

CARL D. CLARKE, Ph.D.
University of Maryland, Baltimore

VOLUME 29
JANUARY—DECEMBER, 1944

ST. LOUIS
THE C. V. MOSBY COMPANY

1944

Copyright, 1944, By The C. V. Mosby Company

(All rights reserved)

Printed in the
United States of America

Press of
The C. V. Mosby Company
St. Louis

The Journal of Laboratory and Clinical Medicine

VOL. 29

JULY, 1944

No. 7

CLINICAL AND EXPERIMENTAL

CORONARY DISEASE ASSOCIATED WITH A SHORT PR INTERVAL AND A PROLONGED QRS

A CASE REPORT

CAPT. S. A. LEADER, M.C., A. U. S.

THE occurrence of a short PR interval with a prolonged QRS has been described many times since it was first reported over twenty-five years ago. Much controversy has arisen as to the mechanism of its production, and there is still considerable dispute. For example, in 1930 Paul White, with Wolff and Parkinson, made a thorough study of a series of cases and ascribed the condition to a functional bundle branch block, but emphasized its occurrence in healthy individuals with normal hearts. In 1938 White stated, "... the impulse in a given case may pass through a short circuit, perhaps a bundle of Kent (Kent, 1893) and sometimes through the usual circuit by way of the bundle of His (Holzman and Scherf, 1932)." In 1933 Wolferth and Wood put forth the explanation of an accessory conduction bundle, known as the bundle of Kent. In 1941 these last two authors presented additional cases in support of their original hypothesis. However, in 1938, Katz and Kaplan reported a case in a patient with severe cardiac damage caused by coronary sclerosis, which they contend renders quite unnecessary the theory of conduction over the bundle of Kent. They maintain that the mechanism consists of an upper nodal rhythm, with a spreading of the impulse through a preferential path in the junctional conduction tissue. Tung, in a report in 1936, concludes that functional bundle block probably of vagal origin occurs and is not a sign of heart disease. Pardee, in his textbook published in 1941, states that the most acceptable explanation is that suggested by Cossio, who contends that these complexes are due to the

Received for publication, Jan. 25, 1944.

excitation of an overirritable focus in the ventricle by the auricular systole. Levine and Beeson, in 1941, described three cases of this type, which manifested paroxysms of ventricular tachycardia, rather than the auricular tachycardia ordinarily found. In one case acute coronary thrombosis was so closely simulated that an erroneous diagnosis was made. Levine mentions another case in which the patient developed paroxysms of auricular fibrillation and went on into congestive failure, even though there was no organic heart disease. Finally in 1942 Butterworth and Poindexter, by electrical means, were able to produce experimentally in the cat and in the dog, complexes of the short PR, wide QRS type at will. In these experiments the current generated by the auricular muscle was picked up by small silver electrodes, placed directly on the surface of the auricle. This current was then amplified several thousand times and used to stimulate one ventricle by means of similar electrodes placed on the ventricular epicardium or in the ventricular muscle. They concluded that the most logical explanation of the syndrome is a ventricular asynchronism, with premature contraction of one ventricle, activated by an abnormal conducting pathway. (Their experiments essentially produced another pathway from the auricle to the ventricle.)

Finally, in 1943, Wood, Wolferth, and Geckler made serial sections of the heart from a patient whose electrocardiogram had shown a short PR and a prolonged QRS (there was an inverted T-1). This patient had died during an attack of paroxysmal tachycardia. Gross examination of the heart was negative but histological studies revealed three muscular connections at the right lateral border between the right ventricle and the right atrium. Two of these bridged a small part of the ventricular cavity during their course. The authors concluded that the demonstration of such structures which should be capable of conducting an impulse to the ventricle furnishes adequate support for the hypothesis of an accessory AV conducting pathway to explain this electrocardiographic anomaly.

It is not intended in this report to enter into the controversy as to the mechanism of this interesting manifestation, but merely to present a case in which coronary disease occurred, without modifying the dominant rhythm of the short PR and prolonged QRS, but at the same time leaving unmistakable evidence of coronary disease. It might be well, however, to enumerate briefly just what is definitely known about the condition, omitting all theories and conjectures. (1) It occurs in the absence of organic heart disease. (2) It occurs most often in young people who are subject to paroxysmal tachycardia (auricular). (3) Occasionally paroxysmal fibrillation occurs in place of the tachycardia and very rarely ventricular tachycardia instead of auricular tachycardia. (4) It has been produced experimentally in animals by setting up an abnormal conduction pathway between the atria and ventricles. (5) It has been abolished by the use of atropine, quinidine and exercise. It may return to normal rhythm spontaneously. (6) It occurs much more often in males than in females.

CASE REPORT

P. K., white male, aged 43 years, was admitted on Jan. 5, 1936, because of severe burns of the right thigh and leg. He had been discharged from the Army for epilepsy and

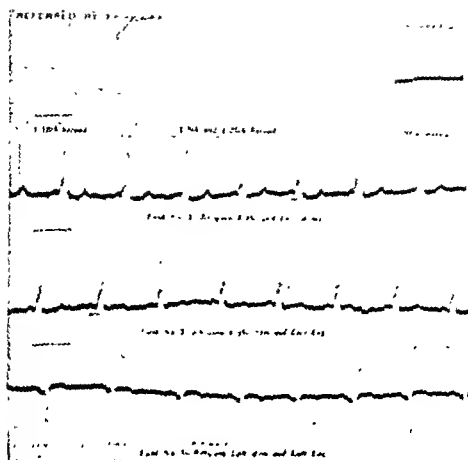


FIG. 1.—2/19/36. No cardiac complaints. Short PR and prolonged QRS.

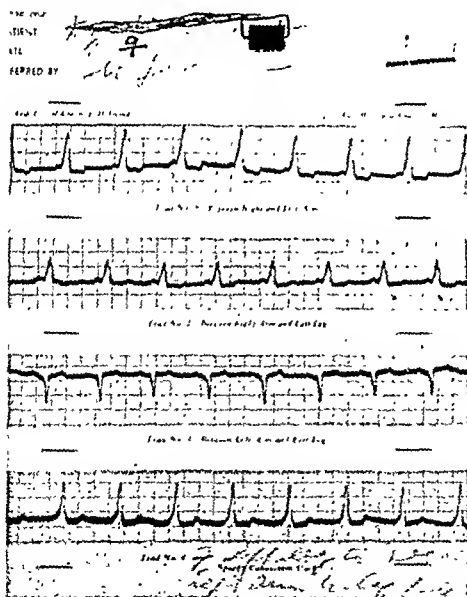


FIG. 2.—9/9/11. After first attack of severe precordial pain. T₁ inverted, ST₁ and ST₂ depressed.

continued to have seizures ever since. Aside from his burns, physical examination revealed only hypertrophic arthritis of the left knee. An electrocardiogram revealed a short PR interval and prolonged QRS (see Fig. 1). The patient had no cardiac complaints, the blood pressure was 90/65, heart rate 70. The heart was not enlarged and there were no murmurs or thrills.

The patient made a satisfactory recovery from his burns, but continued to suffer from his arthritis and to have epileptic seizures. On Sept. 9, 1941, at 8:30 A.M., while stooping over, he was suddenly seized with severe precordial pain, which lasted for three or four minutes, and then subsided but soon became severe again. The severe pain came and went all that day and also the following day, when it was somewhat less severe. The pulse rose to 120 the first day, but soon decreased in rate. There was no temperature elevation. An electrocardiogram taken Sept. 9, 1941 (Fig. 2), revealed, besides the short PR interval and the wide QRS previously noted, a depression of ST-1 and ST-2 and an inversion of T-1. A eardiogram made Sept. 11, 1941, was essentially the same as the first, but a eardiogram taken Sept. 18, 1941 (Fig. 3), showed a diphasic T-2 and T-4.

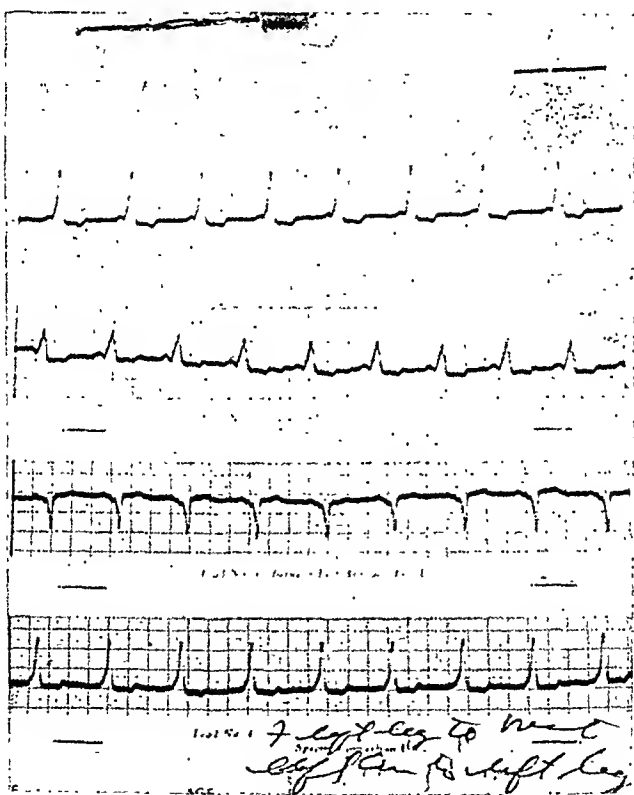


Fig. 3.—9/18/41. T₁ inverted, T₂ diphasic, and ST₁ and ST₂ depressed.

There was no pyrexia or leucocytosis and apparently the patient made an uneventful recovery. He gradually resumed his former activities; for a time he had no cardiac complaints aside from dyspnea on climbing stairs. On Dec. 1, 1941, however, while mopping the floor, he again experienced severe precordial pain, which lasted for one or two minutes, and then eased off. The severe pain was accompanied by a feeling of faintness so that he had to go to bed. Slight pain was still present the next day, but then disappeared. On December 25 he had another attack of severe precordial pain, lasting one or two minutes, but slight pain persisted all day. Following this he had an occasional "twinge" in the precordium, but continued to be up and around.

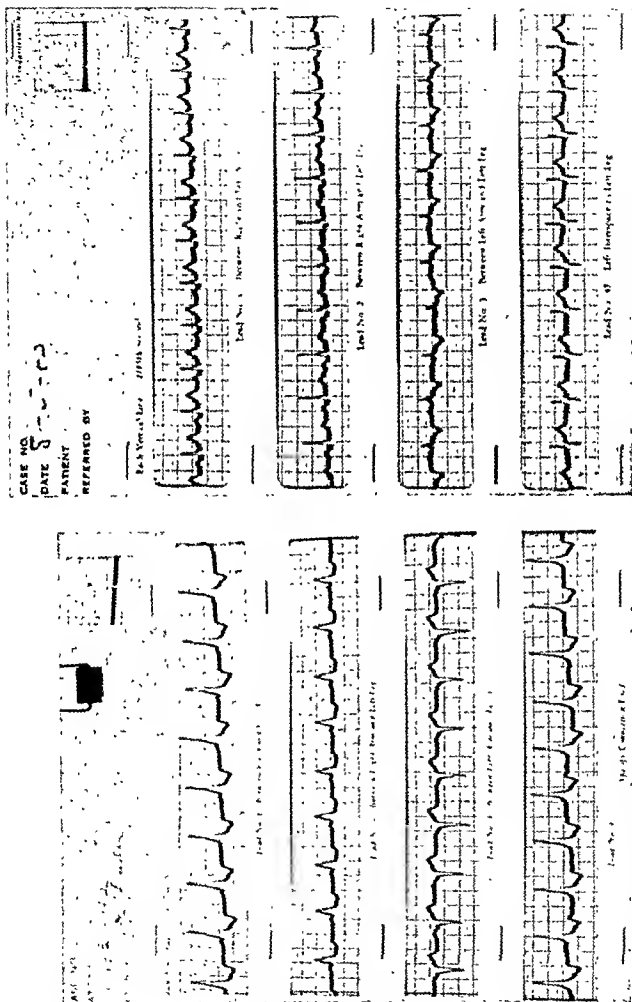


Fig. 4.

Fig. 5.

Fig. 4.—1/21/12. Four weeks after third attack of severe precordial pain. Deep inversion of T₁ and T₂. Diphasic T₃ and depression of ST₁, ST₂, and ST₃.

Fig. 5.—8/5/13. Normal PR and QRS, T₁ and T₂ upright, and ST-1 and ST-2 isoelectric.

On Jan. 21, 1942, an electrocardiogram (Fig. 4) revealed a depression of the ST segments in Leads 1, 2, and 4F, deep inversion of the T-waves in Leads 1 and 4F and diphasic T-waves in Lead 2. The short PR and the wide QRS remained unchanged. Physical examination at this time revealed very little change. The blood pressure was 110/80, the heart rate 102, the white blood count was 11,350, with 70 per cent polymorphonuclears. An electrocardiogram taken Jan. 30, 1942, was essentially the same as the one taken on January 21.

On May 15, 1942, another cardiogram was taken which was similar to that of Jan. 21, 1942. His condition changed little during the next few months. During part of this period he was given theophylline. On March 11, 1943, he had another attack of sharp precordial pain but the temperature and pulse were normal, as were the white blood count and the sedimentation rate. No notable incident occurred during the next few months, but on Aug. 2, 1943, he developed epigastric pain which radiated to the precordium. This pain did not last long, but on Aug. 5, 1943, it recurred. He perspired freely, the sedimentation rate was 30 and the white blood count 16,300. The temperature rose to 102°, pulse to 118. An electrocardiogram, taken on Aug. 6, 1943 (Fig. 5), showed an entirely different pattern for him. Both the PR and QRS were normal in duration and the QRS entirely normal in form. By Sept. 27, 1943, the cardiographic pattern had reverted to its usual form, but T-1 and T-2 were now upright and ST-1 and ST-2 isoelectric, whereas prior to August 6, 1943, T-1 was inverted, T-2 diphasic, and ST-1 and ST-2 depressed (Fig. 4). By Aug. 14, 1943, the patient was able to be up and around, and continues to be at this writing.

COMMENT

Coronary occlusion was strongly considered on the basis of the patient's first severe attack but could never be definitely established, although he developed definite evidence of chronic coronary disease as demonstrated by the tracing of Jan. 21, 1942 (Fig. 4). Again, on Aug. 6, 1943, when all the clinical manifestations pointed to coronary occlusion, the electrocardiogram suddenly reverted to a normal pattern. While the last cardiogram on record still shows a slight depression of ST-1 and ST-4, and diphasic T-1 and T-4, the changes in T-2 and ST-2 present prior to the last episode have disappeared. As mentioned previously, normal rhythm may be restored spontaneously. Just what connection, if any, the patient's last attack had with a transitory return to normal electrocardiographic pattern does not seem apparent.

SUMMARY

A case of a short PR and a prolonged QRS was presented. It was first recorded electrographically over seven years ago. The same rhythm was maintained, even though the patient developed definite evidence of severe coronary disease except for transitory return to a normal pattern during an attack of severe precordial pain.

REFERENCES

1. Ashman, Richard, and Hull, Edgar: *Essentials of Electrocardiography*, New York, 1937, The Macmillan Co., pp. 54-56.
2. Butterworth, J. S., and Poindexter, C. A.: Short PR Interval Associated With a Prolonged QRS Complex, *Arch. Int. Med.* 60: pp. 437-445, 1942.
3. Cossio, P., Berensky, I., and Kreutzer, A.: P-R acortado con QRS ancho y mellado (análisis de las 34 observaciones de la literatura médica, incluyendo 7 observaciones personales), *Rev. argent. de cardiol.* 2: 411, 1936.
4. Glomset, D. G., and Glomset, A. T. A.: A Morphologic Study of the Cardiac Conduction System in Ungulates, Dog, and Man, *Am. Heart J.* 20: 339, 1940.
5. Holzman, M., and Scherf, D.: Über Elektrokardiogramme mit verkürzter Vorhof-Kammer-Distanz und positiven P-Zacken, *Ztschr. f. klin. Med.* 121: 404, 1932.

6. Hunter, A., Papp, C., and Parkinson, J.: The Syndrome of Short P-R Interval, Apparent Bundle Branch Block, and Associated Paroxysmal Tachycardia, *Brit. Heart J.* 2: 107, 1940.
7. Katz, L. N., Kaplan, L. G.: Unusual Forms of Rhythms Involving the A-V Node, *Am. Heart J.* 16: 694, 1938.
8. Levine, Samuel A.: *Clinical Heart Disease*, Philadelphia, 1938, W. B. Saunders Co., pp. 426-427.
9. Levine, Samuel A., and Beeson, Paul B.: The Wolff-Parkinson-White Syndrome, With Paroxysms of Ventricular Tachycardia, *Am. Heart J.* 22: 401, 1941.
10. Moia, B., and Inchauspe, L. H.: Sobre un caso de P-R corto con QRS ancho y mellado presentando asincronismo ventricular, *Rev. argent. de cardiol.* 5: 114, 1938.
11. Pardee, H. E. B.: *Clinical Aspects of the Electrocardiogram*, New York, 1941, Paul B. Hoeber, Inc., p. 132.
12. Pezzi, C.: Consideraciones Patogenicas sur quelques cas de Rhythm Septal et Para-septal Permanents, *Arch. d. mal. du coeur* 24: 1, 1931.
13. Roberts, G. H., and Abramson, D. I.: Ventricular Complexes of the Bundle-Branch Block Type Associated With Short P-R Intervals, *Ann. Int. Med.* 9: 983, 1936.
14. Scherf, D., and Schonbrunner: Beitrage zum Problem der verkurtzten Vorhofkammerleitung, *Ztschr. f. klin. Med.* 128: 750, 1935.
15. Tung, Chen-Lang: Functional Bundle-Branch Block, *Am. Heart J.* 11: 89, 1936.
16. White, P. D.: *Heart Disease*, ed. 2, New York, 1938, The Macmillan Co., pp. 690-691.
17. Wilson, F. N.: A Case in Which the Vagus Influenced Form of the Ventricular Complex of the Electrocardiogram, *Arch. Int. Med.* 16: 1108, 1915.
18. Wolff, L., Parkinson, J., and White, P. D.: Bundle-Branch Block, With Short PR Interval in Healthy Young People Prone to Paroxysmal Tachycardia, *Am. Heart J.* 5: 635, 1930.
19. Wolferth, C. C., and Wood, F. C.: The Mechanism of Production of Short PR Intervals and Prolonged QRS Complexes in Patients With Presumably Undamaged Hearts: Hypothesis of an Accessory Pathway of Auriculo-Ventricular Conduction (Bundle of Kent), *Am. Heart J.* 11: 89, 1936.
20. Wolferth, C. C., and Wood, F. C.: Further Observations on the Mechanism of the Production of a Short P-R Interval in Association With Prolongation of the QRS Complex, *Am. Heart J.* 22: 450, 1941.
21. Wolferth, C. C., and Wood, F. C.: Histologic Demonstration of Muscular Connecting Between Atrio and Ventricle in a Case of Short PR Interval and Prolonged QRS, *Am. Heart J.* 25: 454, 1943.
22. Mnhalm, I.: Wolff-Parkinson-White Syndrome, and Its Pathology, *Helvet. med. acta* 8: 483, 1941.

VERTIGO AND RELATED CONDITIONS*

A NEW THERAPEUTIC CONCEPT

MAJOR MAURICE ELIASER, JR., M.C., A.U.S.

DIZZINESS, giddiness, lightheadedness, sensations of impending syncope, and actual syncope are well recognized as manifestations of cerebral anoxemia. It is also a fairly well accepted clinical fact that the most frequent cause of this anoxemia is cerebral ischemia, which is caused by cerebral arteriosclerosis with or without arterial hypertension. There has been some question as to whether true vertigo with its associated loss of equilibrium and sensation of rotation, with reference to external space, is ever caused by cerebral arteriosclerosis in the absence of thrombosis or rupture of a vessel, although it is admitted that dizziness and lightheadedness are common manifestations of this condition.¹ Actual vertigo, however, has been considered in some instances to be a manifestation of intrinsic vascular disease which may or may not affect the labyrinth.^{2, 3} Lindsay,⁴ in commenting on labyrinthine dropsy, has stated that vasomotor instability may be one of its more likely causes.

Also worthy of consideration is the likelihood that the various symptoms, as mentioned in the foregoing paragraph and which are manifest in the presence of cerebral arteriosclerosis, are caused by a decrease in volume of the blood flow with resultant tissue anoxia. This is analogous to the situation which occurs in angina pectoris and intermittent claudication when the vessels supplying the myocardium and lower extremities, respectively, are involved in occlusive arterial disease. Clinically, it would appear that one of the more important causes of vertigo, giddiness, and allied symptoms in the older age group manifesting arteriosclerosis is cerebral ischemia; this result is due to the sclerotic arterial vessels being insufficiently elastic to compensate for rapid changes of pressure. Especially is this true in regard to the intracranial arteries when the labyrinth is involved; the effects of gravity must be overcome. Also, it is known that only minute degrees of ischemia are necessary to produce symptoms.

The complaints of patients suffering from the syndromes under discussion vary from vertigo and syncope to ill-defined sensations of dizziness and lightheadedness. Characteristically, these symptoms are noted on arising from bed or when suddenly sitting up; they generally follow rapid changes of the position of the head, especially from dependency to the orthostatic position.

It is believed that because of the effects of gravity involved in the venous return from the brain, there may be a disparity between cerebral volume blood inflow and outflow in persons with impaired arterial circulation. This alteration of the normal balance of the circulation⁵ cannot be appreciably improved in

*From the Department of Medicine, University of California Medical School, San Francisco, Calif., and the Cardiovascular-Renal Section, Hoff General Hospital, Santa Barbara, Calif.

Received for publication, Dec. 10, 1943.

organic vascular disease by attempted improvement of the arterial circulation. However, the venous circulation of the intracranial contents is amenable to change inasmuch as the entire venous return is ultimately carried by the jugular veins. Application of certain physiologic principles involved in therapeutic venous occlusion for arterial disease of the extremities⁶ and coronary arteries⁷ to the cerebral circulation seems pertinent. By use of the plethysmograph it has been shown that following venous ligation in the normal, as well as the arterially deficient, extremity, there is produced an increased arterial pulsation which is caused by dilation of the vessels themselves.⁸ It has been demonstrated experimentally that the effect of improved circulation to the part affected is not a humoral or nervous mechanism, but rather a mechanical one. Likewise, it has been shown that venous ligation, as well as the use of pneumatic tourniquet,⁹ produces engorgement of the distal veins and venules which in turn is transmitted through the capillary bed to the arterial tree. This increase in diameter reduces the resistance, and the increased volume of blood flow results in the presence of fixed cardiac output.

For these reasons, it was believed that obstruction in the venous return of the intracranial contents might prove beneficial in the treatment of symptoms attendant upon inadequate arterial blood flow resulting from cerebral arteriosclerosis. With this object in mind, an elastic collar, $\frac{3}{8}$ of an inch in width, was devised with a hook and eye attachment to permit ready removal; it is adjustable and may be tightened sufficiently to cause engorgement of the jugular veins without causing impairment of the arterial circulation. The collar was fitted into place at the base of the neck and well below the thyroid cartilage. The patients who were fitted with this device were advised to put it on fifteen minutes before arising from bed in the morning and to wear it continuously until nighttime when it was to be removed fifteen minutes after retiring.

Current conditions have made it impossible to obtain the clinical records of patients who were previously treated for a statistical tabulation; therefore, only one typical case report will be described.

F. F., male, aged 49 years, had complained of dizziness since October, 1942. The patient entered the hospital on Feb. 9, 1943, prior to which time he had been very active; he had practiced dentistry for twenty years prior to October, 1942. Previously, he had experienced no known arterial hypertension, renal disease, or other major illnesses, operations, or accidents. During the month of October, 1942, he noted the gradual onset of vertigo; this became quite severe within two weeks and necessitated his discontinuing his dental practice. The attacks consisted of recurrent sensations of loss of equilibrium, and subjective rotation, with reference to external space. These symptoms were noted most frequently upon arising from bed in the morning; they usually lasted four to five hours, then gradually subsided. Several of these episodes were induced by the rapid elevation of his head; other times they followed the tying of his shoelaces, or were incurred by leaning over a wash basin. Apart from the attacks of true vertigo, he experienced an almost continuous sensation of unsteadiness in his gait, insecurity while standing for prolonged periods of time, and, occasionally, fear of impending syncope as well as a slightly impaired memory for recent events. There was no associated tinnitus or hypacusis. The pressure was 168/108. The presence or absence of nystagmus at that time was unknown. The condition became progressively more severe, and the patient subsequently transferred to this hospital on Feb. 9, 1943.

Upon physical examination, the patient was observed to be a well-developed, active, 49-year-old man, without edema, cyanosis, or abnormal increase of the venous pressure. Generalized peripheral arteriosclerosis was demonstrable in the temporal, radial, and pedal vessels. The blood pressure was 170/106. The carotid sinus pressure produced no change in the pulse rate, cardiac rhythm, or blood pressure, and was unassociated with any significant symptomatic response. The remainder of the physical examination was not remarkable.

The hemanalysis, urinalysis, and blood Kahn examinations were normal. The phenol-sulfonphthalein excretion totaled 82 per cent in two hours by the intravenous method. Roentgen ray examination of the chest and intravenous pyelography were normal. The electrocardiogram was within normal limits. Following a three weeks' period of observation, during which time various medications were instituted and failed to alter the attacks of vertigo, the patient was fitted with the collar as previously described. Since this time he has been free of dizziness, his sense of equilibrium is normal, and he has returned to work; he now is able to stand for as long as eight hours without a recurrence of these symptoms. In addition to the definite improvement of his memory, he has noted a greatly increased sense of well-being for the first time since the onset of symptoms in October, 1942. The blood pressure has undergone no change since the employment of the collar.

Theoretically, the use of the collar would seem indicated in several conditions other than those under discussion; for example: cerebral arterial thrombosis during its early phase, postural hypotension,^{10, 11} if unassociated with intrinsic arterial disease, and the amelioration of symptoms noted by pilots during rapid descent, the so-called "blackout" sensation.

The only untoward effects provoked by the wearing of this device have been occasional headaches. These apparently are induced by the increased intraeranian pressure which results from having the collar too tight, and they are relieved by reducing the tension of the elastic. The use of the sliding adjuster on the collar, as is employed with garters, has proved to be a valuable contrivance for the maintenance of proper pressure. It has been observed that those patients with mild degrees of involvement are able to remove the collar in the afternoon without incurring a return of distressing symptoms; presumably, this is due to their having become adjusted to the orthostatic position by that time. No effects of carotid sinus stimulation have been induced by the wearing of the collar because of its position at the base of the neck.

As a general rule, the tension of the collar is adjusted so that a demonstrable venous pressure increase is obtained to the extent that venous engorgement is evident to the level of the mandibular angle. It has been found, however, that in fitting the collar to the individual person, the variations in the musculature of the neck, and other so far unknown factors, will require trials to be made with varying tensions before the optimum results are obtained in each instance.

It is realized that there is difficulty in evaluating any therapeutic procedure when the results are dependent upon purely subjective responses and are incapable of objective measurement. The possibility that examination of the retinal veins may be used as an index for establishing the degree of increased venous pressure is to be investigated. However, the accessibility of this device, its inexpensiveness, its lack of production of untoward effects, and its proved value, albeit of a purely subjective nature, in a relatively small group of persons, warrants its employment when other accepted methods of treatment for vertigo and related conditions have failed.

SUMMARY

1. The occurrence of vertigo, giddiness, lightheadedness, and syncope as manifestations of cerebral anoxemia resulting from cerebral arteriosclerosis is described.

2. The rationale of the application of certain principles of therapeutic venous occlusion for these symptoms is explained.

3. The clinical history of a patient who manifested the afore-mentioned syndrome and the results of therapy with partial jugular venous occlusion is reported.

4. Application of the principles involved in other conditions resulting from cerebral anoxemia is intimated.

REFERENCES

1. Weiss, Soma: *The Pharmacopeia and the Physician; the Treatment of Vertigo and Syncope*, J. A. M. A. 118: 529, 1942.
2. Wechsler, Israel S.: *A Textbook of Clinical Neurology*, ed. 5, Philadelphia and London, 1943, W. B. Saunders Company, p. 365.
3. Cecil, R. L.: (Friedman, E. D.) *Textbook of Medicine by American Authors*, Philadelphia, 1941, W. B. Saunders Company, p. 1535.
4. Lindsay, J. R.: Labyrinthine Dropsy and Ménière's Disease, *Arch. Otolaryng.* 35: 853, 1942.
5. Graham, Evarts A.: (Brooks, Barney) *Surgical Diagnosis by American Authors*, Philadelphia, 1930, W. B. Saunders Company.
6. Linton, R. R., Morrison, P. J., Ulfelder, H., and Libby, A. L.: Therapeutic Venous Occlusion; Its Effect on Arterial Inflow to the Extremity, as Measured by Means of Rein Thermoströmuhr, *Am. Heart J.* 21: 721-742, 1941.
7. Gross, Louis, Blum, L., and Silverman, G.: Experimental Attempts to Increase Blood Supply to Dog's Heart by Means of Coronary Sinus Occlusion, *J. Exper. Med.* 65: 91-108, 1937.
8. Lewis, T., and Grant, R.: Reactive Hyperaemia in Man, *Heart* 12: 73, 1925.
9. Collens, W. S., and Wilensky, N. D.: Intermittent Venous Occlusion in Treatment of Peripheral Vascular Disease; Experience With 124 Cases, *J. A. M. A.* 109: 2125, 1937.
10. Ellis, L. B., and Haynes, Florence W.: Postural Hypotension With Particular Reference to Its Occurrence in Disease of the Central Nervous System, *Arch. Int. Med.* 58: 773-798, 1936.
11. Stead, E. A., Jr., and Ebert, R. V.: Postural Hypotension; A Disease of the Sympathetic Nervous System, *Arch. Int. Med.* 67: 546-562, 1941.

REVERSIBILITY OF SENSITIZATION OF ERYTHROCYTES

G. M. KALMANSON, M.S., PH.D., AND J. J. BRONFENBRENNER, PH.D., D.P.H.,
St. Louis, Mo.

IN PREVIOUS studies¹ we have sought to ascertain whether biologic agents which have been neutralized by antibody could be recovered in the active state. In these studies we took advantage of the fact that the proteolytic enzyme papain can apparently completely destroy the antibody function of the antibody molecule.² Thus, when the neutralized agents were submitted to digestion with this enzyme, it was found to be possible to recover them in the active form. By this method we have succeeded in quantitatively "reactivating" neutralized bacteriophage and botulinus toxin. Analogous experiments with intact, fully virulent pneumococci indicated that these organisms could be recovered in the virulent state even after having been completely sensitized, with all the antibody they could adsorb, to the extent of complete loss of infectivity for mice. We have also ascertained that the floccules of precipitate formed by the interaction of type specific polysaccharide of the pneumococcus with its homologous antibody could be dissolved by the action of the enzyme, with the liberation of the specific soluble substance into the medium. Finally, we have found that the pneumococcus "Quellung" reaction could be completely reversed by papain digestion, and the pneumococci thus denuded of their serum coating would again show "Quellung" reaction upon subsequent addition of fresh antibody.³

These studies bring direct supporting evidence to the concept that when an active biologic agent is "neutralized," the agent itself undergoes no essential change, except that it has become coated by antibody and thus cannot exert its characteristic action. When the antibody is removed, the original state of activity is restored.

We were interested in studying by this technique the effect of sensitization of erythrocytes by hemolytic antibody ("amboceptor"). Specifically, we wished to ascertain whether, by analogy with our previous findings, the susceptibility of sensitized erythrocytes to lytic action of complement could be abolished by the digestion with papain.

Technique.—The stock papain solution used in these experiments was prepared as follows: 4 Gm. of papain powder (Sargent) was shaken with 100 ml. of distilled water for one and one-half hours. It was then filtered through paper and diluted to 200 ml. This solution was kept at $\pm 7^{\circ}$ C. under toluene and, when needed, an aliquot portion was activated by adding 0.1 ml. of 16 per cent solution of cystein hydrochloride for each ml. of enzyme solution, the hydrogen ion concentration adjusted to pH = 7.0-7.2, and the mixture incubated at least thirty minutes at 37° C. before use.

Received for publication, Feb. 21, 1944.

From the Department of Bacteriology and Immunology, Washington University School of Medicine.

The digestion of antibody was carried out by adding 0.1 ml. of the activated papain solution to 0.4 ml. of 4 per cent suspension of sensitized red cells and incubating for one hour at 37° C. The free enzyme was then removed by several cycles of washing with physiological salt solution in the angle centrifuge, and finally complement was added in the usual way to determine the presence or absence of sensitization.

Rabbit antishoop amboceptor and guinea pig complement were used throughout these experiments.

Results. In the preliminary experiments it was found that if 0.2 ml. of 4 per cent suspension of red cells, sensitized with from two to ten units of amboceptor, is subjected to digestion with papain, the cells lose their susceptibility to lysis by complement. To control this experiment, the sensitized cells were mixed with comparable amounts of papain which had not been activated, or with cysteine alone, and in either case the cells remained sensitive to complement.

It was noted in the above experiment that when the 0.2 ml. of 4 per cent red cells suspension had been sensitized with 10 units of amboceptor or less, the superambient fluid contained no free amboceptor, indicating that it had all been absorbed by the erythrocytes. We were interested in repeating these experiments under conditions where the red cells were completely saturated with amboceptor, that is, when they had been sensitized by large excess of amboceptor. In order to accomplish this, it was first necessary to ascertain how much amboceptor a standard dose of red cells (0.2 ml. of 4 per cent suspension) could absorb. Thus 0.2 ml. of the red cells was mixed with 10, 20, 40, 80, and 160 units of amboceptor, respectively. The mixtures were then incubated at 37° C. for 30 minutes, centrifuged, and the supernates tested for residual amboceptor by serial dilution method. It was found that the cells present in 0.2 ml. of 4 per cent suspension took up approximately a maximum of 40 units of amboceptor.

The final experiment was then conducted as follows: To each of eleven tubes containing 0.2 ml. of 4 per cent suspension of red cells was added 80 units (large excess) of amboceptor (0.2 ml. of a 1:12.5 dilution). These mixtures were then centrifuged for 15 minutes in the angle centrifuge, and resuspended in 0.4 ml. of physiologic saline. To 8 of the tubes was added 0.1 ml. of activated papain, and the remaining tubes received, respectively, 0.1 ml. of saline, of papain solution which had not been activated, or of cysteine. The mixtures were reincubated for an hour at 37° C. and then washed with saline 5 times (to remove the added enzyme and other substances), and finally the sediments were each resuspended in 0.4 ml. of saline. Part of the tubes (1-4) in which the sensitized cells were digested with activated papain were tested for susceptibility to lysis by various amounts of complement, and part (5-8) were resensitized with various amounts of amboceptor and then tested with one unit of complement. As controls, the sensitized cells not subjected to papain digestion (9-11) were tested with one unit of complement. The results of the entire experiment are given in Table I.

This experiment indicates that when the red cells are sensitized to saturation with an excess of amboceptor, they become resistant to the action of comple-

TABLE I

DESENSITIZATION OF ERYTHROCYTES BY DIGESTION OF THE ANTIBODY WITH PAPAIN AND RESENSITIZATION BY FURTHER ADDITION OF AMBOCEPTOR

TUBE NO.	SENSITIZED CELLS	ENZYMES	TREATMENT	AMOUNT OF COMPLEMENT ADDED	RESULT	AMOUNT OF AMBOCEPTOR ADDED FOR RESENSITIZATION	AMOUNT OF COMPLEMENT ADDED	RESULT
	ml.	0.1 ml.	Incubated one hour at 37° C., then washed 5 times with saline and resuspended in 0.4 c.c. saline	Units		Units	Units	
1	0.4	Active Papain		1	No lysis	-	-	-
2	0.4	Active Papain		5	No lysis	-	-	-
3	0.4	Active Papain		10	No lysis	-	-	-
4	0.4	Active Papain		20	No lysis	-	-	-
5	0.4	Active Papain		-	-	1	1	Lysis
6	0.4	Active Papain		-	-	5	1	Lysis
7	0.4	Active Papain		-	-	10	1	Lysis
8	0.4	Active Papain		-	-	20	1	Lysis
9	0.4	Saline		1	Lysis	-	-	-
10	0.4	Papain not active		1	Lysis	-	-	-
11	0.4	Cysteine		1	Lysis	-	-	-

ment upon digestion of the antibody. This is true even when as much as 20 units of complement are used to induce hemolysis, indicating the completeness of the return to the unsensitized state. These cells can now be resensitized with even a minimum amount of amboceptor (1 unit) so that they become susceptible to hemolysis by but one unit of complement.

These results shed no direct light on the mechanism of the action of complement on the sensitized red cell. However, they do indicate that there has been apparently no permanent change in the cells as a result of the combination with hemolytic antibody as the cells revert to their original state of nonsusceptibility to complement upon the removal of the antibody from their surface.

SUMMARY

Red cells sensitized with an excess of amboceptor may be rendered completely resistant to complement, if the antibody is digested away by means of papain. These previously sensitized cells, after digestion by papain, can now be resensitized with a minimum of amboceptor.

REFERENCES

1. Kalmanson, G. M., and Bronfenbrenner, J.: Restoration of Activity of Neutralized Biologic Agents by Removal of the Antibody With Papain, *J. Immunol.* 47: 387-407, 1943.
2. Kalmanson, G. M., and Bronfenbrenner, J.: The Reactivation of Neutralized Phage and Other Biologic Agents by Digestion of the Antibody With Papain, *Federation Proceedings* 1: 2, 179, 1942.
3. Kalmanson, G. M., and Bronfenbrenner, J.: The Reversal of *Pneumococcus Quellung* by Digestion of the Antibody With Papain, *Science* 96: 21-22, 1942.

NOTE ON THE "DIGESTION" OF METAL IN THE STOMACH*

H. NECHELES, M.D., PH.D., AND W. H. OLSON, CHICAGO, ILL.

THE "digestive" power of the stomach for metals has impressed us for a number of years during our experience with metal cannulae of all types used for the draining of gastrostomies or of pouches (Pavlov or Heidenhain) in dogs. The cannula consisted of a base plate and a tube. The base plate held the cannula in the stomach or pouch, and the tube was protruding to the outside, thus draining the stomach or the pouch. The cannulae were made of brass, ordinary steel, and stainless steel. They were made either of one piece, or the base plate was soldered, screwed, or braised to the tube. Soft, hard, or silver solder was used. A number of cannulae had been plated on the inside and outside with nickel, gold, or chromium. During periods varying between months and years a number of these cannulae dropped out of the stomachs or of the pouches because the solder from the base plate had dissolved; hard solder or silver braising were affected slower than soft solder, but dissolved nevertheless. We, therefore, constructed cannulae from one piece of metal in order to be certain that the gastric juice could not act on the joint between the plate and the tube. These cannulae lasted somewhat longer but were "digested" away eventually. In another number of dogs the base plate had dissolved entirely, or a section of the metal tube above the base plate had been "digested." The metal which apparently was destroyed easiest was stainless steel. On inquiry, the manufacturers told us that stainless steel had little resistance to weak mineral acids and that we would have been better off if we had used ordinary steel. In a number of cannulae a hole was digested through the tube, so that gastric juices leaked out around the tube.

Plating of the cannulae with different metals was not of much value. Apparently, small cracks or small bubbles which had been formed during the plating and which were not visible to the eye led to a diffusion of acid under the plating and to the eventual destruction of the cannula. The most resistant cannulae were those made of brass, but even they eventually became "digested." Apparently there are individual differences between dogs, because some animals might retain their cannulae for many years and in others they might be destroyed within a few months. We cannot explain this difference. The acidity in the pouches of our dogs was high; in many of them near the limits of the physiologic capacity of the stomach to secrete acid. In the case of the gastrostomy dogs, the acidity was naturally lower, because food, saliva, regurgitation, etc., tended to diminish it considerably. However, in these dogs "digestion" of the cannulae occurred.

*From the Department of Gastro-Intestinal Research of Michael Reese Hospital. Aided by a grant from the Nelson Morris Research Fund. The Department is in part supported by the Michael Reese Research Foundation. Received for publication, Dec. 30, 1943.

From the standpoint of the experimentalist it is to be welcomed that Wangensteen and his associates have developed a new cannula from plastic material. We hope that this cannula will stand up better in prolonged chronic experiments than our metal cannulae. We want to bring our experience to the knowledge of other workers in this field as a warning, and to the members of the medical profession in order to demonstrate how powerful the gastric juice is, not only to our ordinary food, but even to strong metal.

THE ADJUVANT EFFECT OF AEROSOL UPON THE GERMICIDAL ACTION OF CADMIUM CHLORIDE

ARTHUR F. COCA, M.D., PEARL RIVER, N. Y.

THE limitation of the fungal causes of ringworm to the epidermis and its appendages has suggested local treatment of this infection with fungicidal agents, and such treatment has been so frequently successful that one could be encouraged in the thought that the complete success of the method could be accomplished through a study of the several factors that conceivably are concerned in it.

In the present study the following pertinent factors have been considered:

1. The selected fungicidal agent should be one possessing a relatively low toxic or irritative property with respect to the cutaneous tissues.
2. The fungicidal agent should possess a sufficient property of diffusion into the cutaneous tissues and into the infective fungi; or if this property of diffusion is not sufficient in the agent itself, nonirritative adjuvants of such property must be sought.
3. If such an agent or mixture has been found it must be applied in a manner that allows sufficient time for effective penetration of the infected tissues and the infective fungi.

In 1935 the fungicidal property of cadmium chloride was strikingly shown when a heavy growth of mold that had resisted an oily antiseptic having a phenol-coefficient of 11 was entirely destroyed by a single application of a 10 per cent aqueous solution of cadmium chloride.

A one per cent solution of cadmium chloride in 20 per cent ethyl alcohol was then used in the treatment of a scaly eczema of the scalp and auditory canals. There was less scaling when the solution was applied daily but the eczematous lesions of the scalp were hardly influenced. The solution was not irritating.

When the marked wetting property of sodium dioctyl sulphosuccinate, Aerosol OT, became known, it seemed likely that its addition to the alcoholic solution of cadmium chloride would favor diffusion through the tissues and possibly penetration of the infective microorganisms by the cadmium salt.

One per cent of aerosol was used and it was found necessary to increase the concentration of the alcohol to 30 per cent in order to keep the mixture in solution.

The application of this solution daily to the scalp and ears in the above-mentioned case soon caused disappearance of the dandruff and healing of the eczema. Thereafter, recurrence of the condition could be prevented by applications at about three-day intervals.

From the Lederle Laboratories, Pearl River, New York.
Received for publication, Feb. 24, 1944.

The same person was affected with recurring ringworm of the forearms and feet (athlete's foot) and these infections were entirely cured and later recurrence could be prevented through appropriate use of the alcoholic solution of cadmium chloride and aerosol. On one occasion the weekly use of the solution on the feet was discontinued for three weeks, after which time the early lesions of athlete's foot suddenly appeared between the second and third toes of the right foot accompanied with an intolerable itching. The condition healed in about three days after local applications of the solution were begun.

On the basis of these encouraging preliminary experiences, a number of cases of ringworm, affecting chiefly the feet, were treated with almost invariable success. Exceptionally resistant were infections of the plantar and palmar skin and the fingernail. Such cases required longer treatment, contact with the solution being maintained for 15 minutes or longer with gauze or cotton wet with the fluid. Clinical details of these cases will be reported elsewhere.

The first experiments designed to demonstrate the enhancement of the fungicidal action of cadmium chloride through the admixture of aerosol were carried out in an aqueous medium. The aerosol was used in a 50 per cent solution in propylene glycol, which was itself fungicidal. The addition of aerosol distinctly, though not markedly, increased the fungicidal action of the cadmium salt. When, however, the tests were carried out in a 20 per cent ethyl alcoholic medium the adjuvant action of aerosol was found to be remarkably strong. This experiment is illustrated in the table.

TABLE I

Illustrating the experiments that show the adjuvant effect of aerosol upon the germicidal action of cadmium chloride. The solutions of cadmium chloride and those of aerosol were made in 20 per cent ethyl alcohol.

The percentile figures of the two substances in the mixtures represent the final percentages obtaining after the mixtures were made. A constant quantity of the respective emulsified* microorganisms was transferred with bacteriological loop to the several mixtures and control fluids and after one-half hour a loopful of each of these was spread over the surface of a suitable nutrient agar slant. The slants were incubated at 37° C. The readings for survival were made after 48 hours.

CdCl ₂ (½%) in each tube	Growth after 48 hours	CdCl ₂ (¼%) in each tube	Growth after 48 hours
aerosol	Monilia albicans	aerosol	Monilia albicans
%		%	
1/16	none	1/16	none
1/32	none	1/32	none
1/64	none	1/64	none
1/128	none	1/128	2
1/256	none	1/256	few
1/512	1	1/512	good
0	good	0	good
Aerosol alone in 20% ethyl alcohol	Monilia albicans		
%			
1/2			
1/4	3		
1/8	good		
1/16	good		
	heavy		

*The well-shaken emulsion of *Monilia albicans* was filtered through a silk fabric.

It is seen that whereas cadmium chloride alone in concentrations of $\frac{1}{2}$ and $\frac{1}{4}$ per cent in 20 per cent alcohol was incapable of sterilizing suspensions of the microorganisms used in the tests, and whereas aerosol alone in the same solvent failed to kill these microorganisms in concentrations of $\frac{1}{16}$ per cent, the same two concentrations of cadmium chloride did sterilize the suspension when mixed with aerosol in concentrations as weak as $\frac{1}{256}$ per cent and $\frac{1}{64}$ per cent respectively.

TREATMENT AND CONTROL OF EPIDERMOPHYTOSIS AND BROMIDROSIS IN A STATE SCHOOL WITH CADMIUM CHLORIDE-AEROSOL SOLUTION

G. W. T. WATTS, M.D., THIELLS, N. Y.

EPIDERMOPHYTOSIS and bromidrosis are two clinical conditions occurring in institutions for which many remedies have been prescribed but for reasons discussed below few have been found entirely satisfactory. Recently, however, Lederle Laboratories of Pearl River, N. Y., made available to Letchworth Village, for investigational use, a cadmium chloride-aerosol solution which they had shown by laboratory and clinical tests to be fungicidal and antiodorant. This solution was given a clinical trial on 100 institutional cases. The results here recorded have shown it to be effective in the treatment and control of both epidermophytosis and bromidrosis, and most practical for institutional use.

This clinical trial was conducted among the adult female group at Letchworth Village, a state school under the New York State Department of Mental Hygiene, situated at Thiells, N. Y. Epidermophytosis and, to a lesser extent, bromidrosis are an ever present medical problem in this institution. These women, for the most part young and able-bodied, work during the day in various parts of the institution such as the laundry, the kitchens, and the infirmaries. Because of the nature of their work and the shoes worn their feet sweat or may be wet a good part of the time. Since such conditions favor the growth of the infectious fungi, it has not been unusual to find 25 per cent or more of patients in a cottage or dormitory afflicted with epidermophytosis of varying degrees of severity.

Prior to the use of the cadmium chloride-aerosol solution, various approved methods of treatment for epidermophytosis had been tried without reducing materially the incidence of the condition. These included such measures as the daily use of foot baths of 1 per cent sodium hypochlorite; daily foot baths of 1:5000 potassium permanganate solution; daily application of Whitfield's ointment U.S.P. full or half strength and in the most severe cases application of 1 per cent aqueous solution of gentian violet.

While effective in individual cases, each of these methods has disadvantages when applied to an institutional group. Foot baths are too time-consuming, particularly when one attendant has to treat a great number of cases in a limited time. Whitfield's ointment, even when used half strength, may cause erythema, burning, and itching in a certain number of cases. Gentian violet has been reserved for severe cases, for, while it gives highly satisfactory results clinically, it is difficult to remove from the hands, from floor or bed clothing and, therefore, is unsatisfactory because of the extra work it causes.

The cases of epidermophytosis selected for treatment with cadmium chloride-aerosol solution were of two general types which we labeled as intertriginous and hyperkeratotic. The first group (intertriginous) comprised 70 recent cases of mild, moderate, or extreme severe. In mild and moderate cases, the skin between the toes was white and with deep fissures occurring frequently. In severe cases, skin was raw and painful.

In these cases the feet were bathed daily, after which the solution was applied freely with a cotton-tipped applicator. In severe cases, the patient was confined to bed and treated with wet dressings of boric acid solution. Patients were able to bear the cadmium chloride-aerosol solution. Treatment continued daily for from two to four weeks, although the itching subsided and the drying effect of the solution observed after a few days. Patients were cured under this regime.

In two instances daily use of the cadmium solution caused redness and itching of the skin. In such cases treatment was discontinued and a 1% iodine ointment was used for a few days. Thereafter, treatment with the cadmium chloride-aerosol solution was given two or three times a week.

The likelihood of reinfection from patients' shoes or from floors used by infected individuals was recognized. Since the use of shoes as advocated by Beckman¹ is impractical for us at present, the shoes were sponged with cadmium chloride-aerosol solution and exposed to sunlight during the day. Since these measures are probably inadequate, the key for prevention of serious recurrence is placed upon the systematic daily inspection of the patient's feet with resumption of treatment when lesions appear.

BROMIDROSIS

To try the value of eadmium-aerosol solution as a deodorant, sixteen female patients who suffered severely from bromidrosis, manifested as odorous underarm perspiration were selected. In all of these cases, even though shaving was performed at monthly intervals, the underarm odor was so strong that it would pervade a moderately sized room in a few minutes.

Each patient treated herself daily. After bathing, the solution was applied under arm with the finger tips. In all cases the results have been highly satisfactory and, where the patient is not doing heavy work, the odor is controlled completely. No complications have resulted. The solution is non-irritating to the skin and does not affect clothing deleteriously.

Similarly six cases were selected who were afflicted with bromidrosis of the feet. Each was put under treatment. The feet were bathed daily after which the solution was applied with a cotton-tipped applicator. In all cases the objectionable odor was completely controlled.

TABLE I

SUMMARY OF CLINICAL RESULTS IN 103 CASES WITH CADMIUM CHLORIDE-AEROSOL SOLUTION

DIAGNOSIS	NO. OF CASES	CURED	IMPROVED
I. Epidermophytosis			
A. Hyperkeratotic	14		14
B. Intertriginous			
1. Slight	38	38	
2. Moderate	26	26	
3. Severe	6	6	
II. Bromidrosis			<i>Controlled</i>
A. Underarm	12		12
B. Feet	6		6
III. Tinea circinata	1	1	

The eadmium ehloride-aerosol solution submitted to the institution for investigational study has been in use for the past seven months. The incidence of cases of epidermophytosis during this time has been reduced markedly. Present indications are that we have here a therapeutic agent by means of which we can effectively reduce the severity and control the spread of epidermophytosis among a group of susceptible patients. Likewise, it is an efficient and convenient antidodorant.

MANUAL AND MECHANICAL RESUSCITATION IN EXPERIMENTAL ASPHYXIA

BERNHARD STEINBERG, M.D., AND ALBERT DIETZ, Ph.D., TOLEDO, OHIO

THE purpose of this work is to determine the relative efficiency of manual and mechanical resuscitation with the type of instrument employed in our experiments. Sufficient reduction of oxygen intake results in severe damage to tissues and eventually in death. Complete restriction is fatal within several minutes. Conditions in which the body oxygen content is reduced are referred to as anoxia. Occasionally, the term is used interchangeably with that of asphyxia. However, the latter designation is applicable to those states which are associated with circulatory failure and retention of carbon dioxide.¹

METHODS AND RESULTS OF INVESTIGATION

Asphyxia was produced by placing a tightly fitting rubber mask over the face of a dog and closing the open end with a rubber stopper. The animals were under sodium pentobarbital anesthesia.

In order to study gross changes in lungs and heart, asphyxia was induced in some animals by excising the anterior part of the chest wall. This produced an equalization of pleural and atmospheric pressures. After variable periods of asphyxia, resuscitation was administered either manually or mechanically with the McKesson resuscitator.

During the experiments, blood pressure tracings were obtained by means of a mercury manometer connected to the femoral artery. Respiratory activity was recorded by placing an inflated rubber tube over the chest and abdomen and connecting it to a tambour. In many instances bronchial respirations were obtained by connecting the tambour to the mask through the medium of a metal tube. Blood was drawn for quantitative analysis of oxygen and carbon dioxide from the femoral or carotid artery. Blood was drawn under oil. The analyses were made by the method of Van Slyke and Neill.²

To study the effects of resuscitation in carbon monoxide anoxia, the gas was administered to animals by a spirometer (with soda lime) which was filled with a mixture of 500 c.c. of carbon monoxide and four liters of oxygen. Carbon monoxide was obtained by the interaction of sulfuric and formic acids. The animals were allowed to breathe the mixture. When maximum respiratory effects were obtained, treatment was begun. This study was done to determine the rapidity of displacement of carbon monoxide by oxygen under various methods of resuscitation. Carbon monoxide analyses were done by the modified method of Van Slyke and Neill² on blood drawn from the femoral artery.

Asphyxia was produced by closing the open end of the mask and after varying intervals of complete cessation of respiratory movements, manual artificial

respiration was applied on four dogs in seven instances (Table I). Quantitative analyses for oxygen and carbon dioxide in arterial blood were made at regular intervals during the asphyxial and recovery periods and correlated with blood pressure and respiratory movements.

TABLE I
ASPHYXIA FOLLOWED BY MANUAL ARTIFICIAL RESPIRATION

NO. OF EXPERIMENT	TOTAL EXCLUSION OF AIR FOR		CESSATION OF RESPIRATORY MOVEMENTS FOR		OUTCOME
	MINUTES	SECONDS	MINUTES	SECONDS	
1	3	31		58	Died
2	4	31		17	Survived
3	5	24		40	Died
4	6	16	1	16	Died
5	6	19	1	2	Survived
6	6	24		46	Survived
7	6	40		23	Survived

Oxygen was quickly depleted from the arterial blood. The gas reached the low content of 0.17 to 1.41 volume per cent in 2 to 7.3 minutes of asphyxia. The total oxygen loss varied from 83.5 to 98 per cent of the preasphyxial level. The carbon dioxide gain was from 3.7 to 10.1 volume per cent. The total increase of carbon dioxide from the preasphyxial level varied from 6.5 to 25 per cent. These variations were due probably to the degree of narcosis, hemoglobin content, acid-base balance, etc. In less than two minutes after manual resuscitation was started, the oxygen content of the arterial blood reached the preasphyxial level. The carbon dioxide decreased below the preasphyxial content.

In instances of recovery, the blood pressure rose fairly quickly, in 18 to 34 seconds after artificial respiration was begun. In some cases when manual

TABLE II
ASPHYXIA FOLLOWED BY ARTIFICIAL RESPIRATION WITH THE RESUSCITATOR

NO. OF EXPERIMENT	TOTAL EXCLUSION OF AIR FOR		CESSATION OF RESPIRATORY MOVEMENTS		RESUSCITATION BEFORE MAXIMUM BLOOD PRESSURE IS REACHED
	MINUTES	SECONDS	MINUTES	SECONDS	SECONDS
1	3	6	1	31	45
2	4	6	--	26	24
3	5	14	1	19	38
4	6	4	1	--	81
5	6	23	1	5	18
6	6	24	--	54	27
7	6	30	1	40	46
8	6	57	1	22	20
9	7	57	2	45	-- ^v
10	8	18	1	20	81
11	8	50	1	12	-- [*]
12	9	2	--	25	33
13	9	45	1	55	-- [*]
14	10	57	1	22	-- [*]
15	11	22	1	37	-- [*]
16	11	50	1	9	34
17	13	28	1	3	125
18	15	30	--	40	105

*Animals died. All others survived.

resuscitation was too brief for the degree of asphyxia, the blood pressure, after an initial rise, fell again. Subsequent manual resuscitation resulted in smaller blood pressure elevations.

Asphyxia was produced eighteen times in eleven dogs in one series of experiments. After variable periods in which complete cessation of respiratory movements was obtained, artificial respiration with the mechanical resuscitator delivering oxygen was instituted (Table II). Quantitative analyses for oxygen and carbon dioxide of arterial blood and records of blood pressure and respiratory movements were obtained.

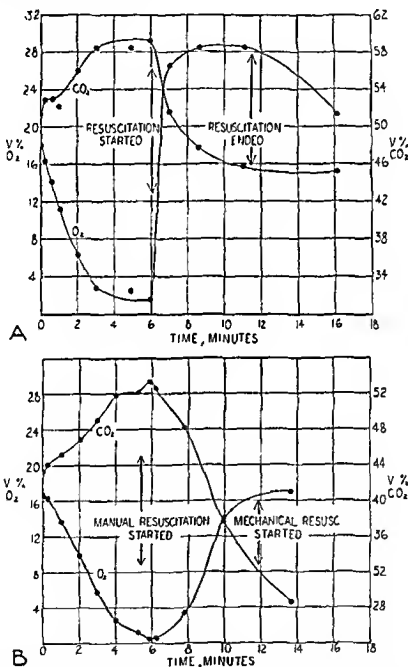


Fig. 1—A, Example of oxygen and carbon dioxide content in the arterial blood during asphyxia and after artificial respiration by a mechanical resuscitator. Note the rapid rise of oxygen to level of asphyxial. The carbon dioxide dropped to a level below pre-asphyxial. The animal died. B, Example of oxygen and carbon dioxide content in the arterial blood during asphyxia and after artificial respiration by a mechanical resuscitator. Note the rapid rise of oxygen to level of asphyxial. The carbon dioxide dropped to a level below pre-asphyxial. The animal survived.

The loss of oxygen and gain in carbon dioxide during the asphyxial periods was essentially similar to that described in the experiments on manual resuscitation. In the stage of recovery, the return of oxygen was more rapid and it reached a higher concentration than in the preasphyxial period. The accumu-

lated carbon dioxide, after resuscitation had been begun, was lost rapidly. The restitution to preasphyxial levels was within the space of a minute. (Fig. 1.)

The maximum blood pressure was reached in surviving animals in from 18 to 105 seconds after resuscitation was begun. The pressure leveled off in the course of a few minutes. The respiratory movements returned to approximately preasphyxial type within the same period.

At no time during the life of any of the animals, did air or resuscitating gas fill the stomach. The organ became distended only when death occurred.

The next series of experiments was performed on seven dogs to obtain a comparison of three methods of removal of carbon monoxide: inhalation of air, inhalation of five per cent carbogen and resuscitation with oxygen. Carbon monoxide was removed most rapidly by inhalation of carbogen. Resuscitation with oxygen was almost as efficient. Inhalation of air lagged considerably behind the other methods. (Fig. 2.)

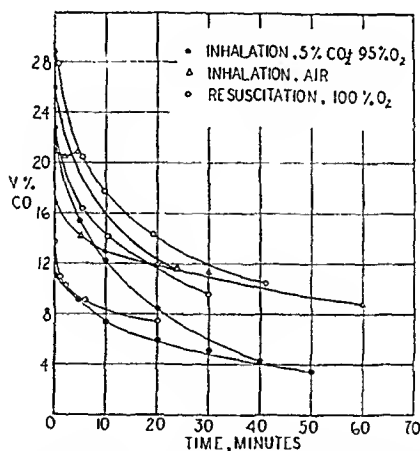


Fig. 2.—Determination of carbon monoxide in the arterial blood following treatment by inhalation of air, inhalation of carbogen, and resuscitation with oxygen. Note the greater efficiency of the latter two procedures.

The McKesson resuscitator and inhalator employed for artificial breathing in our experiments is of the following construction. The machine operates by virtue of compressed gas in the cylinder. The gas pressure in the cylinder in the case of oxygen is reduced to 50 pounds per square inch from a possible maximum of 2,000. Mechanical inhalation and expiration is accomplished by the action of gas under the reduced pressure on a piston which operates a bellows. The gas used to operate the piston is also delivered to the patient. The McKesson machine differs from other similar devices in the following: the volumes of gas delivered in inspiration and removed at expiration are fixed; a part of the expired air is reused; the balance of that air escapes through an escape valve which may be set at varying pressures; an additional fixed safety valve is provided for escape of the air. As a consequence of this mechanism, the lungs are never completely depleted of residual air. There are three controls. One valve controls the rate of resuscitation. Another regulates the volume of gas introduced and withdrawn from the lungs. The third valve controls the pres-

sure of the gas delivered to the patient (Fig. 3). There is also an inhalation device which allows the patient to inhale any volume of gas he desires up to 130 liters per minute.

In order to study the effects of the resuscitator, artificial respiration with the machine was administered to seven dogs under sodium pentobarbital anesthesia for periods varying from one to eight hours. Two of the animals were killed for the study of anatomic changes. One animal had been given artificial respiration for three hours and was killed immediately afterward. The other dog was given artificial respiration for six hours and killed twenty-four hours later. Gross examination of the organs revealed no abnormalities. Histologic study of the organs showed no abnormal changes outside of a mild hyperemia.

Analyses for oxygen and carbon dioxide of the arterial blood were done at regular intervals during the period of artificial respiration. The oxygen content showed an initial increase within the first five minutes, which varied from 3.2 to 6 volume per cent. The gas content in the arterial blood during the rest of the experiment continued more or less at this increased level.

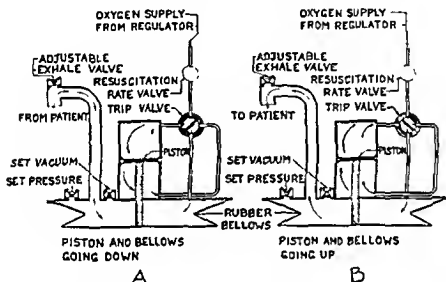


Fig. 3.—A schematic drawing of the McKesson resuscitator device. A indicates gas flow during patient's expiration. B, gas flow during patient's inspiration.

The carbon dioxide content of the arterial blood was decreased during the first 5 to 15 minutes of artificial respiration. The decrease varied from 1 to 5.5 volume per cent. The carbon dioxide content varied during the experiment from the preresuscitation level to that in the first several minutes. Since the resuscitator allows the rebreathing of part of the expired carbon dioxide, it would appear that the gas would be elevated in the blood. However, that was not true. It was apparent that the physiologic regulation limited the gas content. The rebreathing of part of the expired air takes the place of carbon administration.

DISCUSSION AND SUMMARY

Acute asphyxia is a relatively rapid state which extends over a period which seldom exceeds fifteen minutes and which terminates in death.

Oxygen and carbon dioxide exchange constitutes the essential factor in these experiments. Within two to four minutes of acute asphyxia, the blood becomes almost completely devoid of oxygen. To insure recovery of the animal

in acute asphyxia, the oxygen must be restored with a rapidity comparable to its loss. Accumulation of carbon dioxide is simultaneous with loss of oxygen.

Methods in treatment of various degrees of asphyxia or anoxia may be classified into spontaneous and artificial. The former method presupposes the presence of adequate voluntary respiratory movements. The spontaneous method may be further subdivided into inhalation of air or oxygen or oxygen and carbon dioxide mixtures from an appropriate apparatus. The artificial method may be divided into manual resuscitation by various procedures such as Schaefer, Silvester, etc., and mechanical by several current devices. Since recovery of the animals was not attempted until all respiratory movements had ceased, the spontaneous method played no part in our asphyxial experiments.

Artificial manual resuscitation was performed on a number of animals. Recovery was obtained in a fairly large percentage. Recovery, however, was possible only when the duration of asphyxia was relatively short. The return of oxygen content of arterial blood was relatively slow. In some instances, death occurred after recovery from acute asphyxia with the oxygen content of the blood at normal preasphyxial level. It is believed that the cause of death was largely due to the sluggish return of oxygen and production of irreversible tissue damage. Artificial respiration with a mechanical device was responsible for a greater number of survivals. Survival was achieved after a relatively long period of asphyxia. Return of oxygen content to greater than preasphyxial levels was instantaneous.

In recovered animals, normal breathing and circulation was established after a relatively brief period of artificial respiration by either method. With the particular device employed in our experiments, there is a partial rebreathing of the expired carbon dioxide. This serves to add carbon dioxide to the resuscitating pure oxygen. There was no abnormal accumulation of carbon dioxide in the arterial blood nor were any disturbing physiologic effects noted. Mechanical devices have received condemnation from some investigators^{4, 5} and warm support from others.⁶⁻⁹ In our opinion, each method has its value depending upon conditions which call for their employment. When a mechanical device is not available, obviously the manual method must be utilized. On the other hand, availability of a tested mechanical device justifies its use. It is felt, however, that an intimate knowledge of the particular method and device is essential for its most effective use. In these experiments, no damage directly to the lung tissue or indirectly to other organs or tissues was observed even under exaggerated prolonged period of mechanical resuscitation.

CONCLUSIONS

1. Resuscitation with high percentage of oxygen administered in acute asphyxia by a mechanical device used in our experiments resulted in a more rapid return of oxygen content in the arterial blood than in manual resuscitation.

2. Prolonged resuscitation with the mechanical device did not produce abnormal functional or tissue changes in animals.

3. Inhalation of carbon dioxide and oxygen or resuscitation with oxygen and rebreathing of expired air with the mechanical device resulted in a rapid elimination of carbon monoxide.

4. Mechanical resuscitation in acute asphyxia with the device employed in our experiments is a more efficient procedure than manual resuscitation.

REFERENCES

1. Gellhorn, D.: *Anatomic Regulations*. New York. Interscience Publishers, Inc., 1943, p. 54.
2. Van Slyke, D. D., and Neill, J. M.: *The Determination of Gases in Blood and Other Solutions by Vacuum Extraction and Manometric Measurement*, J. Biol. Chem. 61: 523-573, 1924.
3. Peters, J. P., and Van Slyke, D. D.: *Quantitative Clinical Chemistry*. Volume II. Baltimore, Williams & Wilkins, 1943, p. 328.
4. Henderson, Y., and Turner, J. M.: *Artificial Respiration and Inhalation*, J. A. M. A. 116: 1508-1515, 1931.
5. Winters, R. M.: *Methods of Resuscitation*, J. Lab. & Clin. Med. 26: 272-278, 1940.
6. Coryllos, P. N.: *Mechanical Resuscitation in Advanced Forms of Asphyxia. A Clinical and Experimental Study of the Different Methods of Resuscitation*, Surg., Gynec. & Obst. 66: 698-722, 1938.
7. Thompson, S. A., Birnbaum, G. L., and Ostrow, E.: *Resuscitation in Advanced Asphyxia. Role of Positive and Negative Pressure*, Surgery 12: 284-293, 1942.
8. Birnbaum, G. L., and Thompson, S. A.: *Resuscitation in Advanced Asphyxia*, J. A. M. A. 118: 1364-1367, 1942.
9. Martinez, D. B.: *The Mechanical Resuscitation of the Newborn*, J. A. M. A. 109: 489-490, 1937.

BOTULISM FROM HOME-CANNED BEETS

BETTY L. HALL,* LOS ANGELES, CALIF.

WITHIN the last three months, there have been brought to the attention of the Los Angeles City Health Department, two outbreaks of botulism caused by eating home-canned beets.

The case reported herein occurred around Jan. 6, 1944, and the investigation was made by Mr. R. S. Plunkett, R. S., of this department.

As the history of the cases, methods of canning of the beets, their subsequent handling, and the laboratory findings were in accord, we considered the case worthy of a detailed report.

On Jan. 6, 1944, Mrs. S., during the process of concocting some "borscht," removed seven whole beets and a pint of the red beet juice, from a jar of home-canned beets, and, after proper preparation and manipulation, this mixture was added to the other ingredients to complete the above "delicacy." The same day, about 6 P.M. Mrs. S., and her two grandchildren, aged 11 and 7 years, partook of this "borscht."

The following day, the 7-year-old child returned from school at noon complaining of terrific vomiting and retching and, as her grandmother described her, "with her eyes almost popped out." She was taken to the hospital and succumbed the following day with symptoms typical of botulism. The other child, aged 11, also returned from school the same day as the first, about 3 P.M., with similar symptoms; she was sent to the hospital and also died the following day. The grandmother was stricken about 6 P.M. with dizziness, violent vomiting, and blurred vision. She was sent to the hospital, given 15,000 units of botulinus antitoxin and eventually recovered.

Specimens of the beets and some of the borscht was submitted to the laboratory for examination. Animal inoculations, controlled with botulinus antitoxin A and B, showed that the beets and likewise the borscht, contained type A botulinus toxin. Anaerobic cultures were also made from the beets in order to isolate the causative organism. The beets showed a reaction of pH 6.9 which is practically neutral.

In describing the method used in canning the beets, Mrs. S. stated that she had first boiled the beets a few minutes until the skins would peel easily, then reboiled them and put them into the washed half gallon syrup container jars. From the fact that the label was still intact and in good condition on the jar, when received in the laboratory, it was assumed that very little washing and no sterilization was attempted on this container. The amount of cooking described for the beets was patently not sufficient to kill the botulinus spores that must have been present.

Too much stress cannot be laid on the necessity of thoroughly sterilizing all food and containers if attempting to do home canning. The above tragedy is just another example of the danger in the handling of home-canned products.

*Bacteriologist, Los Angeles City Health Department.
Received for publication, Feb. 9, 1944.

THE WATERHOUSE-FRIDERICHSEN SYNDROME

REPORT OF A CASE TERMINATING IN RECOVERY

LT. COL. HAROLD W. POTTER, M.C.

1ST LT. LEWIS H. BRONSTEIN, M.C.

THERE are numerous case reports of the Waterhouse-Friderichsen syndrome in the literature, particularly that dealing with pediatrics. Since the septicemia in the greatest majority of cases is due to the meningococcus, it is to be expected that, in the future, more cases will be reported in the adult population because of the increased incidence of this infection at present. Death has occurred in practically all the cases that have been reported to date. The picture is usually that of an individual who becomes acutely ill with a very high temperature and no physical findings. In a few hours, a marked purpuric eruption is noticed and within 24 to 48 hours, death occurs. Where cultures are taken, the meningococcus organism is the usual one that is recovered. Autopsy reveals hemorrhages into the adrenals as the outstanding finding. The fatal termination is blamed on the acute adrenal insufficiency that occurs with such hemorrhages because the terminal picture is that of circulatory collapse.

Before the days of the sulfonamides, there was little hope of saving such cases, even if the diagnosis were entertained because serum and antitoxin did not clear the body of the meningococci quickly enough to stop the overwhelming infection. The meningococci are so susceptible to the action of sulfonamides, however, that this possibility exists today. In addition, the availability of adrenal cortical extracts and plasma gives one the means of tiding a patient over the period of adrenal damage until sufficient sulfonamides are administered to clear the body of the organism.

In November, 1942, a soldier was admitted to the Station Hospital complaining of headache and fever of one day's duration. He had a sparse purpuric eruption on his body, a moderately rigid neck, and positive Kernig and Brudzinski signs. His temperature was 104°, pulse 120, respirations 30, and blood pressure 118/76. A spinal tap revealed a cloudy fluid which had 61 cells, 98 per cent of which were polymorphonuclear leucocytes. A few extracellular gram-negative diplococci were seen on smear. Meningococci were grown from this fluid and from the blood.

He was immediately given 6 Gm. of sulfadiazine by mouth followed by 1 Gm. every four hours. For the next twelve hours, until 10:00 P.M., he appeared to be doing well, with the temperature dropping. That evening, he seemed to be weak and it was noticed that although his temperature continued to drop his pulse and respirations were increasing. In addition, he was slightly cyanotic. The drug was continued and he seemed to hold his own during the night, though he did appear quite sick. About 6 A.M., he suddenly collapsed, with harsh

breathing, shallow respirations, and a rapid and thready pulse. The Officer of the Day who had followed him throughout the night stated that the death was in the nature of a collapse rather than a toxie death.

An autopsy was performed several hours later. This showed the characteristic central nervous system changes associated with meningitis. There were petechial hemorrhages in the serous membranes and the skin. The lungs and the spleen were markedly congested. The kidneys showed changes of an acute glomerulonephritis and bilateral hemorrhagic necrosis. Both adrenal glands had large areas of hemorrhage in the cortices. Postmortem culture of the spinal fluid resulted in the growth of meningococci. No organisms were grown out of the heart blood.

Although many pathologic findings were seen on autopsy, it was felt that the marked adrenal destruction was responsible for his death. Since this was the first death we had had until then from meningococcal infection and, incidentally, the only one until now, though we have seen more than 80 cases of this disease, we decided to be ready for any subsequent similar case. In preparation for this, we kept adrenal cortex extract on hand and planned to use plasma and sulfonamides in conjunction with this in the next case that seemed to be a candidate for this complication. It was realized that all the medications would have to be administered very early in the course of the disease because the above autopsy showed the numerous pathologic changes that took place in a relatively short time.

The following case report is that of a soldier who was admitted to the hospital four months later with a meningococcal septicemia. He was treated with all the agents mentioned above and recovered after a short and stormy course.

CASE REPORT

This soldier was admitted to the Station Hospital on March 25, 1943, in a confused mental state so that adequate history could not be obtained at that time. He was quite drowsy but could be aroused to perform simple commands, to swallow tablets and fluids. He was evidently acutely ill with a temperature of 103°, pulse of 106, and respirations of 20.

Three days after admission, the following history was obtained from him. He went to bed on March 24, the evening before admission to the hospital, feeling perfectly well. About midnight he had a chill which lasted for several hours. His mouth felt very dry. He was able to fall asleep later and then awoke, feeling very dizzy and extremely weak. A severe headache had developed. This rapidly became worse and he reported for sick call. He had only the vaguest memory of events from that time until about 30 hours after admission.

Physical examination on admission revealed the following pertinent findings. The entire neck, upper chest, and all extremities, particularly the distal portions, were covered with purpuric lesions of all sizes. There were some lesions on the conjunctivae, which were injected. The throat was mildly hyperemic. There was no nuchal rigidity. The blood pressure was 60/40. The spleen was not felt. The Kernig sign was present to a slight degree.

It was felt that he had a fulminating meningococcus infection because sporadic cases had been occurring on the post during that time and because many cases were reported in Baltimore, which was his home. In addition, the rash was similar to that seen in previous cases of meningococcus infection. A blood culture was taken and a spinal tap was done. The fluid was clear and contained two cells, both of which were lymphocytes. The sugar content was 77 mg. per cent. No globulin was detected. Smear of the fluid did not reveal any organisms and none were grown out. The white blood cell count was 11,050 with 2 myelocytes, 22 juvenile granulocytes, 30 "stab" forms, and 39 segmented granulocytes. There were 5 lymphocytes and 2 mononuclear cells. The urine was positive for albumin and had an abundance of granular casts.

He was given 4 Gm. of sulfadiazine intravenously at 1:30 P.M. and 2 Gm. orally at 2:15, 6:00, and 10:00 P.M. Because he had an emesis of 500 c.c. of fluid shortly after the 10 P.M. dose, he was given 2 Gm. intravenously at 3:30 A.M. of March 26. Following this, he received 1 Gm. sulfadiazine orally every four hours. The blood sulfadiazine level at 7 A.M. of March 26, was 17.5 mg. per cent. The level on the morning of March 27, was 18.8 mg per cent.

COMPOSITE GRAPHIC CHART OF CLINICAL RECORD—WATERHOUSE-FRIDERICHSEN SYNDROME



Fig. 1.

He took fluids well by mouth, except for the emesis mentioned above, so that the only fluids given intravenously were those contained in the plasma. The chart shows the amount of plasma which he received. This plasma is stored in 5 per cent glucose solution. The chart also shows the amount of adrenal cortex material given, as well as the time of administration, in relation to the other findings.

Between the time of admission and 1 A.M. of March 26, he had not voided, and only 5 c.c. of urine were removed from the bladder by catheterization. At 4 A.M., he was given 50 c.c. of 50 per cent glucose after hot loin packs had failed to cause further voiding. At 4:30 A.M., he passed 150 c.c. of urine. The chart records the intake and output for the preceding hours.

Chemical analysis of the blood was done at 8 A.M. on March 26 and showed nonprotein nitrogen 75, creatinine 2.6, sugar 93, and chlorides 388 mg. per cent. Examination at this time showed minimal nuchal rigidity, no change in the Kernig sign, and no new purpuric spots. The laboratory reported that a gram-negative diplococcus was growing in the blood culture. This was later shown to be a Group I meningococcus.

The blood chemistry determinations were repeated at 3 P.M. and showed nonprotein nitrogen 66.7, creatinine 2.4, sugar 137, and chlorides 370. He appeared mentally clear and looked to be in much better condition even though the blood pressure was low. In order to play safe, more plasma and adrenal cortical extract and synthetic desoxycorticosterone were given, as shown on the chart. In addition, he was given one salt tablet (2.25 gm.) every four hours as tolerated. He did not take many of these tablets because he became nauseated and they were discontinued after 24 hours. The blood chemistry performed on March 29 revealed the nonprotein nitrogen to be 38, the creatinine 1.5, the sugar 110, and the chlorides 396 mg. per cent.

The urinalysis showed normal findings on March 29 and remained normal throughout his stay in the hospital, although a trace of albumin was found on one occasion while he was still taking sulfadiazine. The white blood cell count returned to normal limits on March 31.

His condition gradually improved with a slow rise in blood pressure, which reached 118/80 on April 19, three weeks after the onset of his illness. This level was maintained throughout the convalescent period. The purpuric eruption had completely disappeared by March 30, but a bilateral conjunctivitis was noticed on that day. This cleared in 6 days with local therapy. It is to be noted that he was still receiving sulfadiazine during the development of the conjunctivitis. He received a total of 69 Gm. of sulfadiazine during his stay in the hospital.

On June 24, three months after the onset of his illness, the efficiency of his adrenal cortex was tested by means of the Cutler, Powers, Wilder test. Clinically there was no evidence of any insufficiency of this organ. The urine volume was 1295 c.c., the total chlorides was 121.4 mg. per cent. This is within the range of normal efficiency.

DISCUSSION

This case differs somewhat from the case which ended fatally. The soldier who died had a definite meningitis, with organisms recovered from the spinal fluid, as well as the blood. The sulfadiazine which he had received was apparently sufficient to sterilize the blood, but not the spinal fluid because postmortem cultures were negative for the heart blood but were positive for the fluid. His condition on admission, however, was apparently better than that of the second patient. This latter patient had much more purpura and a low blood pressure. In addition, he was more toxic as evidenced by the cerebral changes. The first man did not appear to be sicker than the average patient with meningitis that we have seen at this post. The second soldier had only a septicemia with the organism. His spinal fluid cytology and chemistry were normal and no organisms could be grown from it.

It is a matter of conjecture to discuss the amount of pathology in the adrenals that could have been present in the soldier who recovered. It must be assumed that he had more than mere edema of the gland on admission be-

cause of several factors. The blood nonprotein nitrogen was elevated. Despite the fact that he had received 50 Gm. of glucose intravenously during the preceding ten hours and had had practically no urinary output, the blood sugar level was only 93 mg. per cent. The blood pressure rose very slowly to his normal level. In some of the reports of meningococcus infection in the literature in which the patient is acutely ill with a low pressure, this value has returned to normal within one to two days. The slow rise in pressure cannot be attributed to his bed rest since he was in bed all the time that this rather steady rise was recorded. His pressure after he was up and about merely showed some fluctuations from his apparent normal of 118/80. The chloride determination does not help too much since the determination of the end point is not too definite and the change in the various determinations that were made did not correspond with the improvement noted in all the other findings. Unfortunately, we were not equipped to determine the sodium content of the blood.

For the first few weeks after he was allowed to be up and about, he complained of fatigability which could be attributed to his severe illness and his prolonged bed rest. His particular type of infection did not seem to be responsible for this. It was felt that it would be too risky to attempt to measure adrenal cortical function too early in his convalescence. This test was therefore done several days before he was discharged to duty. As is noted above, his response was normal and we must assume that, as far as could be determined, he had restitution of his cortical function.

We did not have too much adrenal cortex extract or desoxycorticosterone at hand to give the massive doses that are advocated for patients with acute cortical insufficiency. We tried, however, to use the material at hand as judiciously as possible. We always kept in mind the fact that he might not have too poor an adrenal function and that we would throw him into a water intoxication syndrome. Since plasma was readily available, we placed more reliance on that substance and gave him a fairly good amount of it. In addition, his fluid intake by mouth seemed to be quite adequate. It was our impression, during our vigil, that he obtained more benefit from the plasma than from the adrenal cortex material. We have no more evidence, however, than mere impression.

A study of the chart shows the very definite trend toward improvement in all his reactions. The temperature dropped gradually, as did also the pulse rate. The respirations became slower. The systolic and diastolic pressures rose with an increase in the pulse pressure. Although he had a very low urinary output during the first eighteen hours, this soon corrected itself. It is impossible to be definite about the role played by the intravenous glucose in starting his kidney function. It could as easily have been fortuitous or due to his general improvement.

It is our feeling that his recovery can also be attributed to the prompt recognition of the possibility of this diagnosis. The dispensary medical officer who first saw him recognized the possibility of a meningococcus infection and he was transferred to the hospital and directly to the contagious disease service within a very short time after he was first seen. The usual delays that occur when a patient has to be transferred from ward to ward were eliminated.

CONCLUSION

A case of meningococcus septicemia that had all the characteristics of a Waterhouse-Friderichsen syndrome is presented. This patient recovered. We feel his recovery was due to the use of plasma and adrenal cortex (both as extract and as desoxycorticosterone) to tide him over the period of adrenal insult; also to the use of sulfadiazine, and to the prompt recognition of the possibility of the diagnosis and the immediate institution of treatment.

CLINICAL CHEMISTRY

THERAPY OF MIGRAINE BY ELECTROLYTES AFFECTING THE BLOOD VOLUME

CARL PFEIFFER, PH.D., M.D.,* ROBERT H. DREISBACH, PH.D., M.D., AND
CHARLES C. ROBY, PH.D.†

HYPERTONIC solutions of various crystalloids¹ have been advocated in the treatment of migraine probably on the theory that the headache was caused by cerebral edema. Large and continuous oral doses of calcium salts²⁻⁴ have also been advocated on the finding that some patients are benefited by this regime. Recently Greenhill and Freed⁵ has used ammonium chloride to induce diuresis and thus prevent the syndrome of premenstrual distress which is characterized by edema, restlessness, and headache. The therapeutic regime for headache advocated by Goldzieher⁶ involves the same therapeutic principle, namely, the mobilization of extracellular fluid.

Previous observations⁷ have shown that migraine, menstrual migraine, and relaxation headache are associated with a relative decrease in the effective arterial blood volume. The purpose of this study was therefore to find an electrolyte or combination of electrolytes which would produce a temporary increase in blood volume.

Extensive studies on diuresis and water balance in dogs and rats⁸ indicated that while both calcium and potassium were diuretic if given alone, when given together diuresis did not occur. The diuretic qualities were completely suppressed when they were administered in the proportion of 3 mols of potassium to one of calcium; a ratio which is approximately the same as that found in the blood plasma for these two ions. It was then shown that along with the suppression of diuresis an increase in the blood volume took place when a combination of these ions was given and that the increase was greater than when an equivalent amount of sodium salts were given. This combination of 3K-1Ca was then tried clinically in migraine.

EXPERIMENTAL

The hematocrit, as determined with Van Allen tubes using heparin as the anticoagulant, was used along with simultaneous serum protein determinations as an index of changes in the plasma volume. The serum proteins were determined gravimetrically after acetone precipitation. The effect of intravenous

From the Departments of Pharmacology of Wayne University and the University of Chicago Medical Schools.

Received for publication, Feb. 9, 1944.

*Lieutenant (M.C.) U.S.N.R., Naval Medical Research Institute, Bethesda, Md.

†Lieutenant (C.W.S.) A.U.S.

and oral Ca-K mixtures (Calcium lactate + KCl, CaCl_2 + KCl) on these two factors was compared with that of equi-molar mixtures of sodium salts (NaCl, Na citrate). (Table I and II.) The intravenous injection of the potassium mixture was carried out slowly and with care because of the pain of injection and the danger of cardiac depression.

TABLE I

COMPARISON OF THE HEMODILUTION OBTAINED BY 3K-1CA MIXTURES WITH THE NA SALT EFFECT. AFTER CONTROL SAMPLE THE MIXTURES WERE INJECTED SLOWLY. THE SECOND SAMPLE WAS TAKEN 20 TO 30 MINUTES LATER (AVE. 26 MIN.)

		CONTROL		26 MIN.	
		PROT. GM. %	HEM. %	PROT. GM. %	HEM. %
Mean of 17 subjects	10 c.c. I.V.				
CaCl ₂ — 0.6%		8.34	45.0	7.69	43.5
KCl — 1.5%					
Mean of 6 subjects					
NaCl — 2.0%	10 c.c. I.V.	7.78	47.3	8.04	46.7

TABLE II

SAMPLES WERE TAKEN AT 30, 60, AND 120 MINUTES AFTER THE ORAL DOSE

	CONTROL		30 MIN.		60 MIN.		120 MIN.	
	PROT. GM. %	HEM. %	PROT. GM. %	HEM. %	PROT. GM. %	HEM. %	PROT. GM. %	HEM. %
Mean of 8 subjects								
2.0 Gm. orally								
Ca Lactate-KCl Mixture								
Mean of 5 subjects	8.34	48.2	8.12	47.8	7.92	47.1	7.85	47.1
2.0 Gm. Na Salts Orally	7.92	48.5	7.95	47.8	7.94	47.9	7.86	47.9

The data indicated regularly a greater hemodiluting effect with K-Ca combinations than could be accounted for by the control NaCl salt effect. It was then decided to try this therapy on an extensive scale in migraine. Accordingly, a questionnaire was prepared covering the salient points in the diagnosis of the syndrome, the concomitant symptoms, and important predisposing factors. This study was started in December, 1939, and over 200 patients have now been treated. Adequate histories and follow-up records were obtained on 150 of these 200 cases. The data are analyzed and presented for their value as a statistical study of the migraine syndrome itself as well as an evaluation of the electrolyte therapy employed.

RESULTS

The symptoms of diagnostic importance are summarized in Table III. While the familial history of migraine is about equal in both sexes, it is of interest that the incidence of visual symptoms is significantly greater in the male than in the female. That the incidence of gastric disturbance is greater than the incidence of visual symptoms was also noted by Von Storch.⁹ He found also that the ratio of male to female patients is about 1 to 3. In our series it is 1 to 2.8

TABLE III
OCCURRENCE OF SYMPTOMS OF DIAGNOSTIC IMPORTANCE

	FEMALE PATIENTS 110		MALE PATIENTS 40	
1. Familial history	72	65%	26	65%
2. Visual symptoms*	36	33%	22	55%
3. Nausea and emesis	87	79%	28	70%
4. Hemicrania	41	37%	17	42%

*Severe photophobia included.

Data on minor factors in the migraine syndrome were obtained by direct questioning. The questions with the percentage of positive answers were as follows: Does excitement or nervous fatigue produce headache? (66%) Does going too long without food cause headache? (53%) Does relaxation (such as sleeping late) cause headache? (50%) Any increase in urine flow before or during headache? (33%) Any chocolate intolerance? (27%) Any carbohydrate intolerance? (19%) Does lack of customary coffee cause headache? (19%) Do attacks occur only at menstruation? (25% of females) Other questions regarding hulinia or irritability preceding the headache were also asked. Many patients volunteered answers, but they were not sufficiently concise to warrant tabulation and analysis. Seventeen out of eighteen of the patients who had become pregnant were free of headache during the second and third trimesters. Of these 17 patients several described a gradual return of their migraine in the postpartum months, while the majority had a return of their symptoms within one month. Two had severe migraine headaches several days postpartum. One patient was temporarily relieved by the development of thyrotoxicosis, one by catarrhal jaundice, and one by fever therapy. The results of therapy are tabulated in Table IV.

TABLE IV
DURATION OF MIGRAINE IN RELATION TO THERAPEUTIC RESULTS

THERAPEUTIC RESULTS	A		B		C		D	
Female (110)	23	21%	46	42%	30	27%	11	10%
Average years' duration	14		21		20		19	
Male (40)	13	32%	13	32%	10	25%	4	10%
Average years' duration	20		12.5		18		21	
Male and Female (150)	36 patients		59 patients		40 patients		15 patients	
	24%		39%		27%		10%	

A = Complete relief of migraine headaches.

B = Occasional headaches which now respond to acetyl salicylic acid.

C = Some effect but still require ergotamine tartrate therapy.

D = Therapy not effective.

The patients were treated by administration of the following formula:

Calcium lactate	308 Gm. = 1 Mol. equi.
Potassium chloride	225 Gm. = 3 Mol. equi.

These ingredients were mixed thoroughly in a mortar and packed into No. 0 capsules. When hand packed the capsule content was approximately gr. x (0.650 mg.). All of the patients were observed for a period of at least three months, and many of them have been observed for more than two years.

The most effective regime of therapy was found to be a gradual increase in dosage from gr. x of the mixture the first day to gr. xxx daily of the mixture at the end of one week. At the end of one month the patients were instructed to take only gr. x daily and to increase the dose by gr. xx every hour at the first sign of an impending headache. An increased, colorless urine flow was frequently found to precede the symptoms as a first sign of headache. It should be noted that the patients had well-established migraine histories and that success of the therapy was independent of the number of years the patients had had the disorder. As is usual in any salt therapy, relapses were sometimes encountered if the patient took large doses of the mixture over a period of several months. These were avoided by decreasing the dosage to a bare minimum at the end of one month. That these relapses were not due to the initial success which frequently accompanied any new medication was shown by the facts that: (1) substitution of NaCl and calcium lactate placebo capsules for the Ca-K mixture resulted in marked diminution in the therapeutic effect, and (2) a cessation of therapy precipitated attacks in many individuals and a return to the medication was again attended by at least a month's success.

TABLE V

THERAPEUTIC RESULTS IN 150 CASES ACCORDING TO SYMPTOMS AND PREDISPOSING FACTORS

	TOTAL CASES*	A	B	% A + B†	C	D	% C + D
1. Excitement	99	30	42	73%	22	5	27%
2. Starvation	79	24	31	70%	20	4	30%
3. Relaxation	75	21	36	76%	16	2	24%
4. Preceding diuresis	50	9	25	68%	14	2	32%
5. Chocolate intolerance	40	11	14	63%	14	1	38%
6. Carbohydrate intolerance	28	9	10	68%	9	0	32%
7. Caffeine withdrawal	29	8	14	76%	7	0	24%
8. Menstruation	27	5	12	63%	9	1	37%

*Number of patients with this symptom or finding.

†Percentage with A or B results (see Table IV). Seventeen out of 18 patients had their migraine relieved by pregnancy. One patient for a period of four months during an attack of catarrhal jaundice. One year presumably by thyrotoxicosis since the headache for a period of one subtotal thyroidectomy.

Table V is an analysis of the data from the standpoint of predisposing factors to ascertain whether any one type of case responds more favorably than another. The relation of site of headache to the result of the therapy is interesting in that if the excellent and good results are pooled for the first three categories, the number of cases of right-sided headaches improved is much smaller than the percentage improvement for the left-sided and frontal headaches (38 per cent for right compared to 73 per cent and 76 per cent for the left and bilateral localizations, respectively). This finding may be of significance as an aid in prognosis and selecting patients for treatment.

The mixture of calcium lactate with potassium chloride greatly reduces the fleeting gastric pain frequently induced by the administration of crystalline potassium chloride alone. Less than 5 per cent of the patients noted any gastric sensation from the encapsulated mixture. Only one patient had to discontinue the treatment because of gastric irritation. Some patients ascribed an increased feeling of well-being and warmth of the extremities to the medication. A shortened menstrual cycle was noted in a few. Some patients reported a mild

but consistent cerebral stimulation and an increased ability to stand psychic trauma. No toxic symptoms were noted in the entire series. Several patients reported that a dull generalized headache occurred when too many capsules (10 to 20 per day) were taken. This is probably similar in etiology to the premenstrual headache encountered in some patients. It is relieved by drinking water to the point of diuresis or by the ingestion of caffeine. It is not relieved by acetylsalicylic acid.

TABLE VI

THE EFFECT OF THERAPY IN RELATIONSHIP TO THE INITIAL LOCALIZATION OF THE HEADACHE.
149* CASES

	A	B	C	D
Left frontal or temporal 26 = 17%	9 35%	10 38%	6 23%	1 4%
Right frontal or temporal 32 = 21%	5 16%	7 22%	13 41%	7 22%
Bifrontal or bitemporal 66 = 44%	17 26%	33 50%	13 20%	3 5%
Occipital 15 = 10%	4 27%	5 33%	3 20%	3 20%
Generalized 9 = 6%	2 22%	1 11%	5 56%	1 11%
Vertex 1		1		

*One case not localized.

DISCUSSION

The results of this form of therapy are in accord with the hypothesis set forth as a result of our previous studies,⁷ namely, that migraine is perhaps due to uncompensated fluctuations in the effective arterial blood volume. The hypothesis accounts for many of the vagaries of the migraine syndrome, namely, the onset at puberty and frequent relief at the menopause (correlating with the onset and waning of cyclic activity of the sex hormones with their salt and water retaining properties, the increased incidence in the female over the male due to the greater salt and water retaining power of estrogens over androgens¹⁰) and relief of migraine by pregnancy where a normal concomitant increase in the blood volume obtains. Actual proof of this hypothesis must await experimental methods whereby the effective arterial blood volume can be measured and correlated with cardiac output and peripheral vascular tone. It must also be assumed that an underlying anatomical or physiological familial defect exists which allows vascular spasm or dilation with changes in the effective arterial blood volume.

The suggested salt mixture is certainly more effective than are calcium, potassium or sodium salts when given alone. It has the disadvantage of all salt therapy that some patients become tolerant after several months of use. No attempt was made to reduce NaCl intake during the period of treatment. Also the patients were not restricted in their use (when necessary of acetylsalicylic acid, phenacetin, or ergotamine tartrate). No toxic reactions were observed in the use of these common drugs with the salt mixture. The salt mixture should be given at the first sign of a headache since it can only be given orally. The patients who consistently awaken with a migraine syndrome so severe that

vomiting prevents oral retention of the capsules are, of course, not benefited by therapy unless it is taken prophylactically at bedtime.

Based on the findings of Graham¹¹ and also Atkinson¹² that nicotinic acid is of value in functional disorders, we have recently been treating a series of patients with the calcium-potassium mixture to which 1 per cent nicotinamide has been added. The preliminary results seem even more promising than the use of the salt mixture alone.

SUMMARY

The results of therapy of migraine over a period of three years with a mixture of calcium lactate and potassium chloride are reported. Of 150 patients 24 per cent had excellent results, 39 per cent good results, 27 per cent fair results (still require ergotamine tartrate) and in 10 per cent the therapy was noneffective. The detailed study revealed that spontaneous diuresis may precede the scotoma as the initial sign of an impending attack. Relaxation or "let down" in activity may be a precipitating factor in the migraine syndrome. In this series a better therapeutic response was obtained in patients with left-sided headaches than in those with right-sided headaches.

REFERENCES

1. Villey, G., Buvat, F. J., and Buvat-Pochon: *Rôle des solutions hypertoniques dans le traitement de la migraine*, *Rev. Neurol.* **70**: 32, 1938.
2. Bassoe, P.: *Migraine*, *J. A. M. A.* **101**: 599, 1933.
3. Conwell, D. V., and Kurth, C. J.: *Clinical Approach to the Migraine Problem*, *J. Kansas M. Soc.* **41**: 413, 1940.
4. O'Sullivan, M. E.: *Present Day Status of Migraine Therapy*, *Endocrinology* **24**: 414, 1939.
5. Greenhill, J. P., and Freed, S. C.: *Mechanism and Treatment of Premenstrual Stress With Ammonium Chloride*, *Endocrinology* **26**: 529, 1940.
- Greenhill, J. P., and Freed, S. C.: *Electrolyte Therapy of Premenstrual Stress*, *J. A. M. A.* **117**: 504, 1941.
6. Goldzieher, M. A.: *Endocrine Aspects of Headaches*, *J. LAB. & CLIN. MED.* **27**: 150, 1941.
7. Pfeiffer, C., Dreisbach, R. H., Roby, C. C., and Glass, H. G.: *The Etiology of the Migraine Syndrome—A Physiological Approach*, *J. LAB. & CLIN. MED.* **28**: 1219, 1943.
8. Pfeiffer, C., Roby, C. C., and Smith, R. B.: *Diuretic Effect of Potassium, Calcium, and Magnesium Given Orally in Salt Solution*, *Am. J. Physiol.* **134**: 729, 1941.
9. Von Storch, T. J. C.: *Migraine Syndrome; Comments on Its Diagnosis, Etiology and Treatment*, *New Eng. J. M.* **217**: 247, 1937.
10. Harrop, G. A., and Thorn, G. W.: *Influence of Sex Hormones on Salt and Water Metabolism*, *Tr. Assn. Am. Phys.* **52**: 164, 1937.
11. Graham, J. W.: *Radiation Sickness; Treatment With Nicotinic Acid*, *J. A. M. A.* **113**: 664, 1939.
12. Atkinson, M.: *Observations on Etiology and Treatment of Ménière's Syndrome*, *J. A. M. A.* **116**: 1753, 1941.

CARDIOTOXIC SUBSTANCES IN THE BLOOD AND HEART MUSCLE IN UREMIA (THEIR NATURE AND ACTION)*

W. RAAB, M.D., BURLINGTON, VT.

CARDIAC failure is a common event in the terminal stages of renal insufficiency and uremia. While its manifestations are often overshadowed by other uremic signs, such as central nervous disturbances, vomiting, hemorrhages, etc., it can be considered as the ultimate cause of the fatal outcome in the majority of cases. Dyspnea and attacks of pulmonary edema are particularly conspicuous (Gouley; Luisada; and others) and also peripheral edema appears frequently in advanced stages. Pulsus alternans (Heitz; Chahier and Contamin), tachycardia, and bradycardia are often observed.

Marked alterations of the electrocardiogram, mainly of the "anoxic" type (flattening or inversion of the T waves, depression of the S-T interval) are frequently present in uremia (Wood and White; Beeher; Gouley; Agnoli and Bussa).

Severe degenerative myocardial lesions have been found to be a characteristic of the uremic heart (Merklen and Rabé; Küllbs; Lüscher; Gouley; Solomon, Roberts, and Lisa) and a diminished myocardial creatine concentration was observed in the failing heart muscle of uremic persons by Gross and Sandberg.

All investigators agree in the assumption of an unknown "toxin" as being the cause of myocardial damage and failure in uremia. The serum of dogs with experimental uremia and the serum of uremic patients contain unknown substances which exert a "digitalis-like" toxic effect on the isolated frog heart (Mason, Resnik, Minot, Rainey, Pileher, and Harrison), and cause anoxic changes of the electrocardiogram of the guinea pig, according to Agnoli and Bussa who studied the effects of intraperitoneal or intravenous injection of crude serum or of serum dialysates.

The possibility that these substances may belong to the group of phenols was mentioned by Mason and his associates who observed a cardio-inhibitory effect of phenol and paraeresol on the isolated frog heart. Retention of phenols in the blood as a regular feature of uremia was reported by Beeher, by Marcelongo, and by Diekes.

Another characteristic anomaly of blood composition in uræmia was described by the writer (Raab¹), namely, the presence of excess amounts of substances with a catechol nucleus (benzene ring with two free hydroxyl groups in ortho-position). Such substances are pyrocatechol and a number of catechol

*From the Division of Clinical Medicine, University of Vermont, College of Medicine, Burlington, Vt.

Received for publication, Jan. 7, 1944.

compounds, including adrenalin, sympathin, adrenalone, dihydroxyphenylalanine, epinine, leucoadrenochrome, and others. The colorimetric method used for the quantitative determination of these substances in blood and tissues (Raab^{1, 2, 3}) includes also ascorbic acid by virtue of its two adjacent hydroxyl groups on a lactone ring but, owing to its weak color intensity, ascorbic acid forms only a fraction of the colorimetric results and, in general, does not constitute a seriously disturbing factor.

The term "AC" will henceforth be used, standing for "adsorbable chromogens" (not "adreno-cortical" compounds, as in some previous publications when the colorimetric results were erroneously interpreted as being due to a combination of adrenalin with cortical steroids).

By far the highest AC concentrations were encountered in the adrenal medulla, and extensive experimental series have shown that the AC concentration in blood and tissues is largely dependent on adrenal secretion and probably also on peripheral sympathin production (Raab,^{1, 2, 3} Cannon and Lissák). It appears reasonable, therefore, to consider the adrenal medulla and the peripheral sympathetic "adrenergic" nerve endings as the source of the bulk of AC material, consisting of adrenalin and related substances, which is present in blood and tissues in physiologic as well as pathologic conditions.

The heart muscle, although containing rather low AC concentrations under standard conditions, is characterized by an outstanding tendency to accumulate both artificially administered catechol compounds and spontaneously secreted AC substances (Raab^{1, 4}). Accumulation of excess AC concentrations in the heart muscle beyond a certain critical level was found to be fatal in experimental animals, leading to cardiac failure and death (Raab¹). Also in the majority of patients who had died from heart failure, the total AC concentration or the concentration of adrenalin (sympathin) proper in the myocardium was found above the upper limit of normal (Raab¹).

The striking toxic effects of excess amounts of the most powerful catechol compound, adrenalin, on the electrocardiogram, and on metabolism, structure, and function of the myocardium are well known and were recently reviewed by the author (Raab¹). Prolonged action of adrenalin upon the heart increases the sensitivity to inhibitory stimuli of the vagus nerve (Langecker; Sollmann and Barlow¹) and adrenalin concentrations stronger than 1:10⁶ exert a directly inhibitory toxic effect upon the heart muscle (Sollmann and Barlow²). Other catechol compounds, such as adrenalone, produce effects similar to those of adrenalin on the heart and, although weaker than adrenalin (Tainter; Tani), seem to intensify the efficiency of the latter (Kato and Aibara). Catechol proper (pyrocatechol) acts as a cardiac depressant (Tainter).

In view of the above-mentioned facts, the behavior of AC substances in the blood and in the heart muscle of uremic patients was studied. It was correlated with the electrocardiogram and the clinical picture, and comparative experiments with uremic serum and with various catechols and phenols were carried out on the isolated frog heart and on the intact rabbit.

TABLE I

CASE	AGE	SEX	DATE*	X.P.N., MG. PER 100 G.C.	BLOOD CREATININE MG. PER 100 G.C.	URINE		RED BLOOD COUNT (MILLION PER CU. MM.)	MENTAL CONDITION	ACT. COE. UN. PER C.C.	H.S.T.†	BLOOD PRESSURE	CARDIAC SYMPTOMS	ORIGIN OF UREMIA	COMPLICATIONS
						SPECIFIC GRAVITY	ALBUMIN								
1	37	♂	5/ 4/40	260	4	1008-10	Trace	3.6	Normal	562	1.01	160/ 90	Dyspnea, palpita- tions, pulmonary congestion	Congenital polycystic kid- ney	
2	45	♀	4/22/42	80	-	Almost anuric	+++	-	Drowsy	156	1.00	190/102	Dyspnea, marked edemas	Acute glomeru- lonephritis	Hematuria
3	34	♂	4/25/42	100	-				Drowsy	270	1.17	151/ 98	Dyspnea, marked edemas		
			7/13/42	125	9	Anuric	-			152	1.18	130/ 70	None	Acute nephritis (mercury poi- soning)	Dermatitis
			7/24/42	180	10	1007-20	50%	5.0	Normal	257	1.00	146/ 92	Sl. ankle edema	Chronic glomer- ulonephritis	Sinusitis
4	10	♀	8/ 4/42	72	-		Trace			85	1.21	-	None		
			12/ 4/40	333	27	1008-12	7-19%	1.9	Drowsy	420	1.00	135/ 85	None		
5	8	♀	3/ 5/41	100	8	1005-15	15-30%	3.1	Sl. drowsy	362	1.00	162/ 90	Marked edemas, dyspnea	Chronic glomer- ulonephritis	Epistaxis
6	39	♂	12/ 6/39	104	-	1013-16	5-12%	4.0	Normal	304	-	230/140	Sl. dyspnea, cyanosis, palpitations	Chronic glomer- ulonephritis	Silicosis
7	20	♂	12/ 2/40	185	0	1004-10	Tr.-13%	3.6	Sl. drowsy	283	1.07	165/ 90	None (edema of face)	Chronic glomer- ulonephritis (lead?)	
8	64	♀	5/ 6/43	150	5	1014-15	12-25%	3.8	Drowsy	310	1.10	222/104	Dyspnea, marked edemas, tachycar- dia, hemorrhagic cough	Chronic glomer- ulonephritis	Subcutaneous hemorrhages
9	26	♀	6/ 7/43	123	6	1035-07	Trace	3.1	Drowsy	475	1.03	224/120	Dyspnea, palpita- tions	Chronic glomer- ulonephritis	
10	17	♂	11/25/42	228	5	1008-12	Tr.-2%	4.2	Sl. drowsy	176	1.10	126/ 84	Slight dyspnea	Chronic glomer- ulonephritis	Foot injury
11	11	♀	11/25/42	100	4	1010-11	Tr.-1%	4.1	Normal	146	1.11	130/ 90	None	Chronic glomer- ulonephritis	

TABLE I—CONT'D

CASE	AGE	SEX	DATE*	N.P.N. MG. PER 100 G.C.	BLOOD CREATININE MG. PER 100 G.C.	URINE		RED BLOOD COUNT (MILLION PER CU. MM.)	MENTAL CONDITION	ACT. COE. UN. PER C.C.	D.S.R.†	BLOOD PRESSURE	CARDIAC SYMPTOMS	ORIGIN OF UREMIA	COMPLICATIONS
						SPECIFIC GRAVITY	ALBUMIN								
12	26	♀	1/22/43	220	23					290	1.02	154/115	Moderate dyspnea and edemas, hemor- rhagic cough	Pyelonephritis	
			6/ 2/43	-	-	1009-16	6-13%	1.9	Drowsy	381	1.03	150/120	Moderate dyspnea and edemas, hemor- rhagic cough		
			3/ 8/43	275	6					310	1.14	152/116	Moderate dyspnea and edemas, hemor- rhagic cough		
			3/27/43	229	-					620	1.14	-	Moderate dyspnea and edemas, hemor- rhagic cough		
13	53	♀	11/25/42	320	-	1012-16	Trace	3.8	Deep coma	660	1.02	132/ 90	Tachycardiac	Pyelonephritis	Pneumonia, epilepsy
14	33	♀	4/13/43	153	8	1009-13	14%	3.0	SL. drowsy	543	1.03	172/122	Dyspnea, tachycar- dia, ankle edema	Pyelonephritis (after preg- nancy)	Subcutaneous hemorrhages
15	38	♀	9/10/41	680	21	1006-10	2-4%	3.8	Excited	362	1.10	160/100	Edemas, moderate dyspnea	Obstructive pyelonephritis	Ca. of uterus (pelv. m e- tast.)
16	60	♀	8/ 6/42	196	2	1010-25	Tr.-27%	3.8	Deep coma	315	1.14	172/ 80	Cheyne-Stokes respi- ration, edemas	Obstructive pyelonephritis	Uterus pro- lapse, dia- betes
17	50	♂	4/27/40	270	-	1009-11	18%	3.0	Irrational	322	0.85	190/ 98	Orthopnea, marked edemas	Obstructive pyelonephritis	Prostatic hy- pertrophy
18	76	♂	11/ 8/41	133	-	1007	24%	1.7	Drowsy	265	-	130/ 80	Dyspnea, edemas	Obstructive pyelonephritis	Prostatic hy- pertrophy
19	64	♂	1/23/41	364	17	1008	8%	2.0	Coma	-	-	138/ 78	Dyspnea, edemas	Obstructive pyelonephritis	Prostatic hy- pertrophy
20	72	♂	4/27/43	142	9	1009-16	Trace	4.0	Irrational	-	-	184/100	Cheyne-Stokes respi- ration, hydrothorax	Obstructive pyelonephritis	Carcinoma of prostate

METHODS

1. AC in blood and heart muscles was determined by the modified method of Shaw, as described by the writer (Raab¹). The results are expressed in color units per cubic centimeter or gram, each unit corresponding to the color intensity of 10^{-6} mg. of adrenalin, which was used for standard comparison. The "d.s.r." (denominator of specific ratio) permits a rough evaluation of the qualitative composition of the bulk of AC material found. A "d.s.r." of 2 or more indicates the prevailing or exclusive presence of adrenalin and/or sympathin; lower d.s.r.'s indicate the presence of other related catechol compounds, such as adrenalone, dihydroxyphenylalanine, leucoadrenochrome, etc. An occasional d.s.r. below 1.0 can be assumed to be due to a relatively large amount of ascorbic acid.

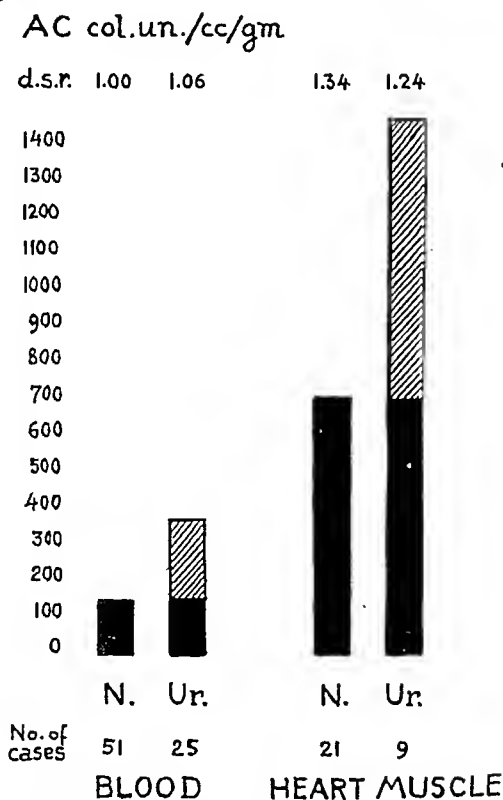


Fig. 1.—Average concentrations of catechol compounds (AC = adsorbable chromogens) in the blood and heart muscle of normal and uremic persons.

2. The frog heart experiments were carried out with hearts of *Rana pipiens*, prepared according to the Straub method, suspended in a moist chamber in an oxygen atmosphere and filled with Ringer's solution which was replaced by fresh serum or various experimental solutions.

3. Alcoholic serum extracts were prepared as follows: 50 c.c. of serum (mixed with an equal amount of 95 per cent alcohol for disinfection) were dried forty-eight hours in a flat plate at a temperature of 37 to 38° C. The

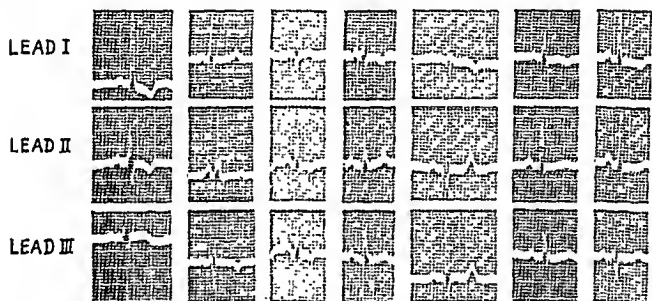
dried serum was finely pulverized in a mortar and extracted two hours in a Soxhlet apparatus with 250 c.c. of absolute alcohol. The alcohol was then evaporated with suction over a hot water bath and the extract cautiously boiled down to a volume of $2\frac{1}{2}$ c.c. This brownish lipid containing residue was kept in small vials in the dark.

4. The rabbit electrocardiograms were taken with a Siemens Atlanco apparatus under nembutal anesthesia.

CATECHOL COMPOUNDS IN THE BLOOD, AND CARDIAC MANIFESTATIONS IN UREMIC PATIENTS

Twenty-eight uremic patients, each with a N.P.N. concentration of at least temporarily 100 mg. per 100 c.c. or more, were examined (Table I). In 19 of these patients the blood creatinine was determined. It was 4 mg. per 100 c.c. or more in 18 cases.

ECG'S OF UREMIC PATIENTS WITH HIGH BLOOD AC.



CASE No.	24	3	23	18	7	17*	6
AGE, SEX	53 ♂	54 ♂	46 ♀	76 ♂	20 ♀	50 ♂	39 ♂
BL. PRESS.	220/110	146/92	188/120	130/80	165/90	190/98	230/140
PULSE RATE	63	58	128	100	65	79	93
N.P.N.	217	180	132	133	185	270	104
BLOOD AC	227	257	260	265	283	302	304
(D.S.R.)	-	(1.00)	(1.64)	-	(1.07)	(0.85)	-

* HAD RECEIVED 18 GR. (TOTAL) OF DIGITALIS ON 2 PRECEDING DAYS.

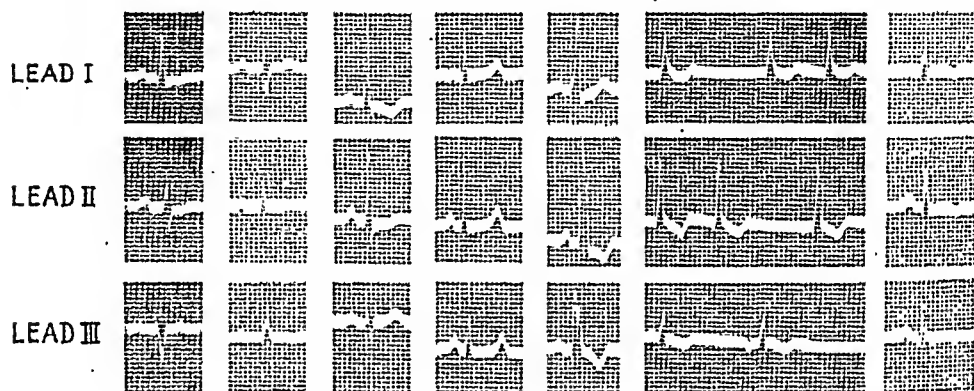
FIG. 2.

In all but three cases (Nos. 10, 11, and 24) the blood AC values were abnormally high (Fig. 1), the normal average (51 cases) being 156 col. un./c.c. ranged between 0.85 and 1.21, thus indicating the prevailing presence of and the highest level of normal being 222 col.un./c.c. (Raab).⁷ The d.s.r. catechol compounds not identical with either adrenalin or sympathin, a condition analogous to the normal.

Signs of cardiac failure were present in the majority of all cases: dyspnea (partly Cheyne-Stokes respiration) (21 cases); pulmonary edema and/or cough with bloody sputum (7 cases); peripheral edemas (16 cases); tachycardia and/or palpitations (10 cases); bradycardia (3 cases). Twenty-one cases were hypertensive. Twenty-three cases were mentally disturbed, drowsy, irrational, or comatose.

Death occurred within a few hours to a few weeks after examination in 19 cases; the further fate of 7 cases is unknown to the writer; two (Cases 3 and 27) improved very considerably and have survived for several months so far, despite high N.P.N., creatinine, and AC values at the time of examination.

ECG'S OF UREMIC PATIENTS WITH HIGH BLOOD AC.



CASE No.	8*	5	21	4.	26	1	12
AGE, SEX	64 ♀	8 ♀	52 ♂	16 ♀	25 ♂	57 ♂	26 ♀
BL. PRESS.	202/104	162/90	230/126	135/85	220/140	160/90	152/116
PULSE RATE	107	88	89	87	108	±80	88
N.P.N.	150	100	270	333	133	260	275
BLOOD AC	310	362	556	420	558	562	620
(D.S.R.)	(1.10)	(1.00)	(1.05)	(1.00)	-	(1.01)	(1.14)

* DAILY 1½ GR. OF DIGALEN

Fig. 3.

In 25 cases electrocardiograms were taken (Figs. 2-4). In 12 the axis position was normal, in 9 there was a left, in 4 a right, axis deviation. Only 5 were free of alterations of the "anoxic type" (Cases 3, 4, 10, 11, 14, and 16). In 20 cases there was flattening or inversion of the T waves (of T₁ alone seven times; of T₁ and T₂ four times; of T₂ and T₃ four times; of T₁, T₂, and T₃ five times). The S-T interval was depressed in 9 cases (S-T₁ twice; S-T₁ and S-T₂ three times; S-T₂ alone three times; S-T₁, S-T₂, and S-T₃ once). In 2 cases (Nos. 15 and 18) S-T₂ and S-T₃ were slightly elevated. The P-R interval was 0.20 or more in 4 cases (Nos. 5, 8, 20, and 22) and 0.12 in one case (No. 23). Digitalis medication was being used at the time of the examination in 4 of the cases with anoxic electrocardiograms (Nos. 2, 8, 9, and 17).

No definite relationship between the degree of AC elevation above normal and the degree of electrocardiographic changes could be established but it appears significant that the only 2 cases with clearly normal AC values (Nos. 10, and 11) had also normal electrocardiograms, and that in 3 cases in which the electrocardiogram was examined repeatedly (Nos. 3, 12, and 24) the anomalies of the T wave appeared or became markedly accentuated as the blood AC level rose.

ECG.s OF UREMIC PATIENTS WITH HIGH BLOOD AND MYOCARDIAL AC



CASE No.	22	15	2*	16	20	28	13
AGE, SEX	39 ♂	38 ♀	45 ♀	60 ♀	72 ♂	65 ♀	53 ♀
BL. PRESS.	238/144	160/100	190/102	172/80	184/100	187/118	152/90
PULSE RATE	97	83	88	81	94	115	125
N.R.N.	267	680	100	196	142	112	320
BLOOD AC	367	362	270	315	-	460	660
(D.S.R.)	(1.00)	(1.03)	(1.10)	(1.14)	-	(1.00)	(1.02)
HEART AC	870	1109	1324	1577	1631	2019	2092
(D.S.R.)	(1.50)	(1.10)	(1.28)	(1.80)	-	(0.98)	(0.99)

* DAILY 4% GR. OF DIGITALIS

FIG. 4.

AC IN THE HEART MUSCLE OF UREMIC PATIENTS AND OF EXPERIMENTAL RATS

In 9 of the uremic cases in which an autopsy* was performed the heart muscle was examined for its AC content (Table II, Fig. 1). In 21 normal (nonfailing) human hearts previously examined, the average AC concentration per gram of myocardial tissue (area near the angle of the descending left coronary branch) was 698 col.un./Gm. Assuming about 1,000 col.un./Gm. as

*The autopsy findings were furnished by the Department of Pathology, University of Vermont.

TABLE II

ABNORMALLY HIGH AC CONCENTRATIONS IN THE UREMIC HEART MUSCLE (THE NORMAL AVERAGE IS 698 COL.UN./GM.)

CASE	WEIGHT OF HEART (GM.)	AC OF HEART MUSCLE (COL. UN./GM.)	D.S.R.	MICROSCOPIC FINDINGS
2	300	1,324	1.28	Negative*
13	250	2,092	0.99	Not examined
15	500	1,109	1.10	Negative
16	350	1,577	1.80	Not examined
19	—	1,463	0.98	—
20	500	1,631	—	Marked fragmentation and light staining of fibers, edema
22	500	870	1.50	Generalized fibrosis, cells poorly staining, striations not distinct
25	700	1,242	1.34	Not examined
28	240	2,019	0.98	Not examined

*Macroscopically the myocardium appeared pale and soft, and the right coronary artery was almost occluded.

the upper limit of normal, it can be stated that in 8 out of 9 uremic cases the myocardial AC concentration was markedly elevated above normal, and in the one remaining case (No. 22) it was above average and not far below the upper limit of normal. The d.s.r. varied, as in normal hearts, between 0.98 and 1.80, thus indicating a prevalence of catechol compounds not identical with either adrenalin or sympathin (except in the one instance with a d.s.r. of 1.80 in which almost pure adrenalin or sympathin seemed to be present).

TABLE III

DEPOSITION OF INJECTED CATECHOL COMPOUNDS IN THE HEART MUSCLE OF THE RAT

SUBSTANCE INJECTED	RAT	DOSE PER GM. OF BODY WEIGHT (MG.)	INTERVAL BETWEEN INJECTION AND DEATH (MIN.)	MODE OF DEATH	AC (COL. UN./GM.)	D.S.R.
Pyrocatechol (d.s.r. 1.00)	1	0.05	20	Killed	1,561	1.00
	2	0.1	20	Killed	3,035	1.10
	3	0.5	5	Spontaneous	18,301	0.91
	4	1.7	2½	Spontaneous	38,759	1.00
Adrenalone (d.s.r. 1.06)	5	0.25	20	Killed	3,654	1.12
	6	0.25	20	Killed	4,040	1.24
	7	0.25	20	Killed	3,776	1.12
	8	0.75	20	Killed	6,818	1.00
	9	1.50	19	Spontaneous	11,167	1.01
Dihydroxyphenylalanine (d.s.r. 1.06)	10	0.15	20	Killed	4,962	1.07
	11	0.75	20	Killed	14,596	1.03

In order to gather some information as to which catechol compounds beside adrenalin (which had been studied previously in this respect, Raab¹) are absorbed by and deposited in the heart muscle, large doses of pyrocatechol, adrenalone, and dihydroxyphenylalanine were injected subcutaneously into white rats, and the myocardial AC concentration was subsequently examined (Table III). The results indicate clearly that the heart muscle possesses an

outstanding tendency to absorb and accumulate not only adrenalin, as demonstrated elsewhere, but also other related catechol compounds, their d.s.r. remaining practically unchanged. The increase of AC in the heart muscle within a limited time was roughly proportionate to the injected doses:

	Pyrocatechol	Adrenalone	Dihydroxyphenylalanine
Dosages:	1 : 2 : 10 : 34	1 : 3 : 6	1 : 5
Increase in heart AC:	1 : 2.5 : 18 : 38	1 : 2 : 3.3	1 : 3

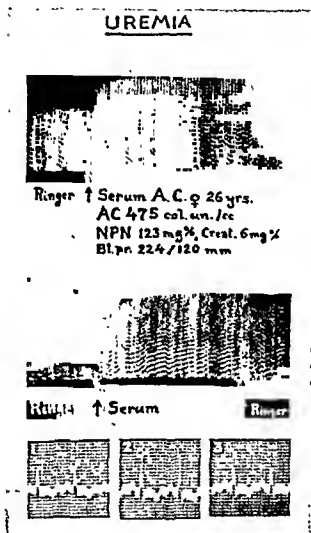


Fig. 5.—Effect of uremic serum on the isolated frog heart. Type 1: Increase of amplitude. The electrocardiogram is that of the patient whose serum was used in this experiment (Case 9).

EFFECT OF UREMIC SERUM AND OF CATECHOL COMPOUNDS UPON THE ISOLATED FROG HEART

The effect of fresh uremic sera upon the isolated frog heart was compared with that of the fresh sera of 20 nonuremic persons, 9 of them hypertensive and 11 nonhypertensive. All nonuremic sera caused a slight to moderate increase in amplitude, ranging from +7 per cent to +28 per cent in the nonhypertensive and from +6 per cent to +38 per cent in the hypertensive cases. Significant alterations of the rhythm were not encountered. The effect of uremic sera, on the other hand, was essentially of three types: (1) A very marked increase in amplitude, ranging from +48 per cent (Fig. 5, Case 9) to +1250 per cent (Fig. 6, Case 8) and even resuscitation of the nonbeating heart (Fig. 6) to extremely vigorous contractions. This effect was produced by the sera of the

UREMIA

R ↑ Catechol 1mg/cc ↑ Serum



Fig. 6.—Effect of uremic serum on the isolated frog heart. Type 3: Combination of increased amplitude with periods of cardiac standstill. Electrocardiogram of the patient whose serum was used in this experiment (Case 8).

uremic cases (Nos. 8, 9, and 27). It cannot be attributed to preceding digitalis medication since it was present also in Case 27 in which no digitalis had been given. (2) Marked bradycardia and periods of cardiac standstill without any significant change of amplitude (Figs. 7 and 8, Cases 12 and 14). (3) A combination of increased amplitudes and periodic standstills (Fig. 6, Case 8). Replacement of the uremic serum by Ringer's solution abolished the bradycardia almost immediately but the amplitude-increasing effect persisted somewhat longer.

UREMIA

ii Serum D.M.♀ 27 yrs. AC 620 col.un./cc, NPN 275 mg/%, Creat 6.0 mg/%

Serum (continued)

↑ Ringer

Ringer ↑ Serum

↑ Ringer

Ringer Serum

↑ Ringer

Fig. 7.—Effect of uremic serum on the frog heart. Type 2 a: Gradually increasing bradycardia without significant increase of amplitude. The electrocardiogram is that of the patient whose serum was used (Case 12)

Attempts were made to reproduce some of the above-named effects of the uremic serum upon the isolated frog heart by introducing various catechol compounds other than adrenalin dissolved in Ringer's solution. Increases of the amplitude, although only of a very slight degree, were produced by adrenalone and dihydroxyphenylalanine, while pyrocatechol (catechol proper) diminished the amplitude and caused persistent cardiac standstill (Table IV and Fig. 9).

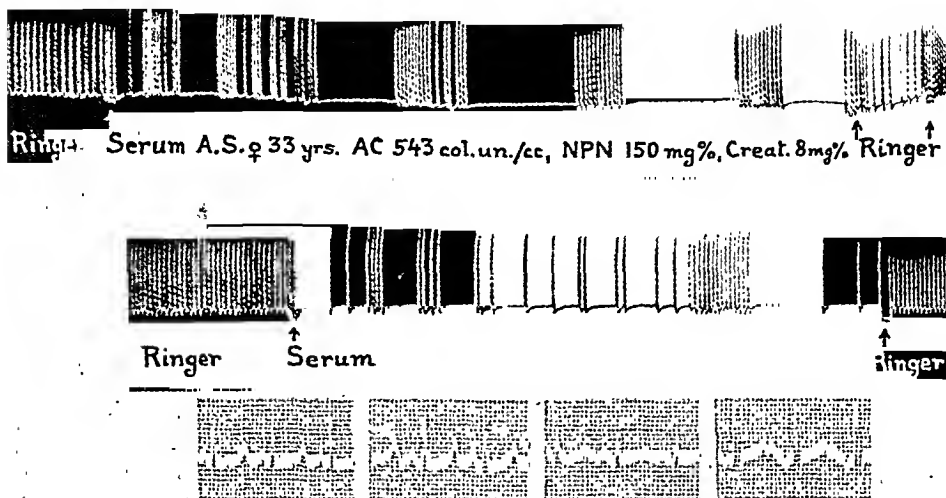
UREMIA

Fig. 8.—Effect of uremic serum on the frog heart. Type 2b: Bradycardia and periods of cardiac standstill without significant increase of amplitude (Case 14). The effect is immediately abolished by Ringer solution.

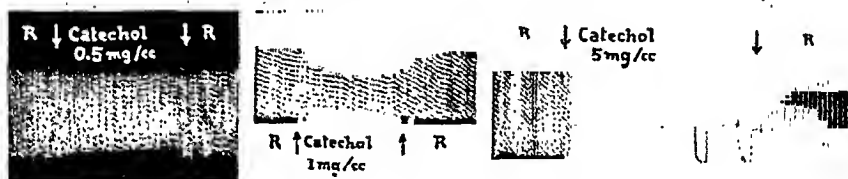


Fig. 9.—Effect of various catechol (pyrocatechol) concentrations on the isolated frog heart.

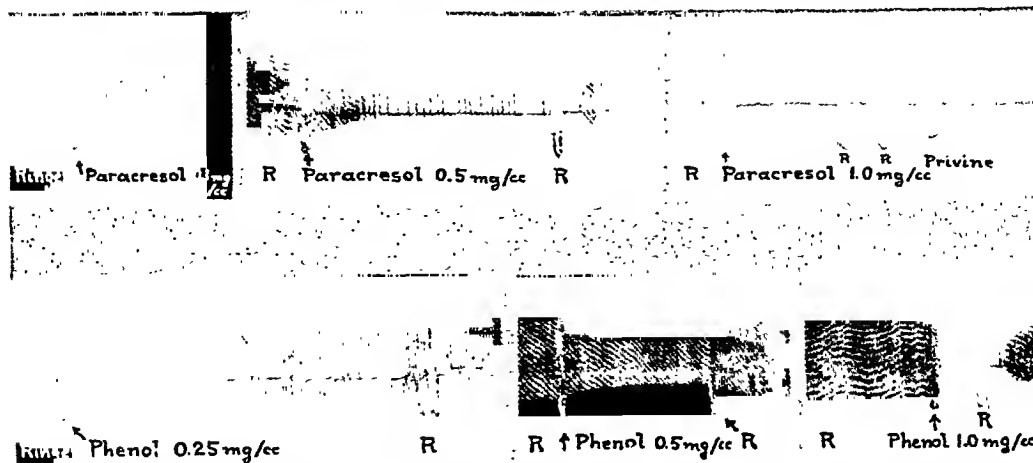


Fig. 10.—Effect of various concentrations of phenols on the isolated frog heart.

TABLE IV

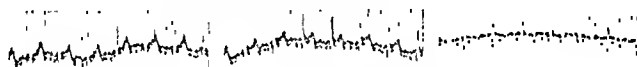
EFFECT OF CATECHOLS AND PHENOLS ON THE AMPLITUDE OF THE ISOLATED FROG HEART

SUBSTANCE	CHANGE OF AMPLITUDE THROUGH MG. OF SUBSTANCE PER C.C. OF SOLUTION							
	0.02	0.1	0.25	0.4	0.5	1.0	2.0	5.0
Adrenalone	+7%	+9%	—	—	+19%	—	—	—
Dihydroxyphenylalanine	—	—	—	+11%	—	—	+19%	—
Pyrocatechol	—	—	-6%	—	-16%	-41%	—	-100%
Phenol	—	—	-8%	—	-44%	-100%	—	—
Paracresol	—	-10%	—	—	-67%	-100%	—	—

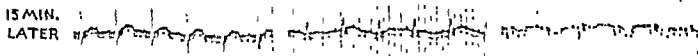
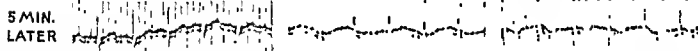
Carbolic acid (phenol) and paracresol as representatives of the phenol compounds, which are also regularly increased in the uremic serum, were likewise examined regarding their effect upon the frog heart. According to dosage they caused diminution of amplitude, bradycardia, and/or periodic or lasting cardiac standstill (Table III, Fig. 10).

RABBIT. - I.V. INJ. OF ALCOHOLIC EXTRACTS OF BLOOD SERA.

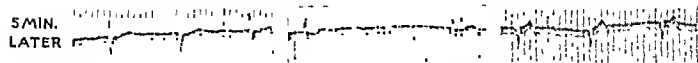
BEFORE INJECTION:



NORMAL SERUM (EXTRACT OF 20 cc.):



UREMIC SERUM* (EXTRACT OF 20 cc.) [30 min. later]



* ♀ 26 yrs. - BLOOD AC 475 col.un./cc., NPN 123 mg%, B.L.P. 224/120 mm.



Fig. 11.—Effect of normal and of uremic serum upon the rabbit's electrocardiogram. (Intravenous injection of alcoholic serum extracts) Death followed three minutes after the last electrocardiogram

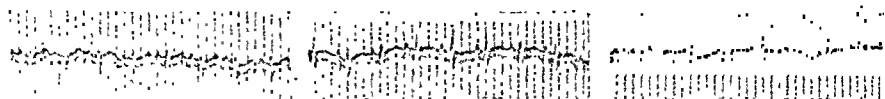
EFFECT OF ALCOHOLIC EXTRACTS OF UREMIC SERUM AND OF CATECHOL COMPOUNDS UPON THE RABBIT'S ELECTROCARDIOGRAM

In order to test the effect of the cardiotoxic constituents of uremic serum upon the rabbit's electrocardiogram, it was necessary to eliminate all protein

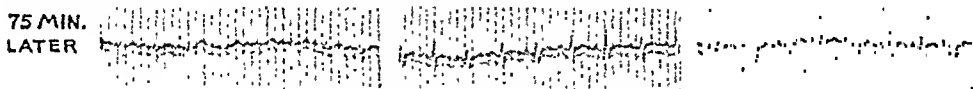
material. Catechol compounds which are otherwise insoluble in organic solvents can be extracted from tissues in the presence of lecithin with chloroform and alcohol (Kendall; Raab³). The quantitative recovery of the total AC from serum by alcoholic Soxhlet extraction had been ascertained before through colorimetric analysis (Raab⁴).

RABBIT. - I.V. INJ. OF ALCOHOLIC EXTRACTS OF BLOOD SERA

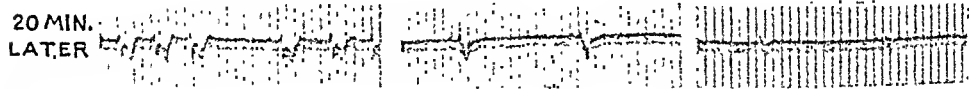
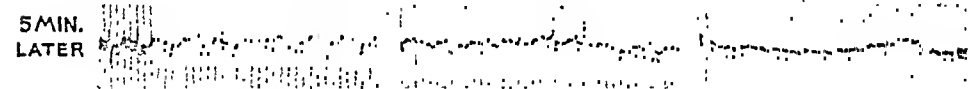
BEFORE INJECTION:



NORMAL SERUM (EXTRACT OF 10 cc.):



UREMIC SERUM* (EXTRACT OF 10 cc.):



* ♂ 34 yrs. - BLOOD AC 305 col.un./cc, NPN 172 mg%, BL.Pr. 215/130 mm.

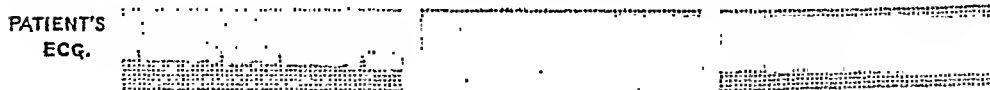


Fig. 12.—Effect of normal and of uremic serum upon the rabbit's electrocardiogram. (Intravenous injection of alcoholic serum extracts.) Death followed two minutes after the last electrocardiogram.

One-half to 1 c.c. of alcoholic extract (corresponding to 10 to 20 c.c. of serum) was diluted with a double volume of physiologic saline solution and injected slowly into the ear vein. (Injection of 1 c.c. of undiluted extract from normal serum had resulted in the death of one animal.) Extracts of normal serum (Figs. 11 and 12), although causing moderate tachycardia or bradycardia and some flattening of the T waves, did not produce any other grossly recognizable effects. The electrocardiograms returned toward normal

within an hour or so. Subsequent injection of equal amounts of uremic serum extract (obtained from Cases 9 and 27) was followed within a few minutes by severe anoxic changes of the electrocardiogram (Figs. 11 and 12), flattening and inversion of the T wave, depression of the S-T interval, preterminal bradycardia, and death within eight to twenty-two minutes.

EFFECT OF CATECHOLS AND PHENOLS, 50 MIN. AFTER S.C. INJ.

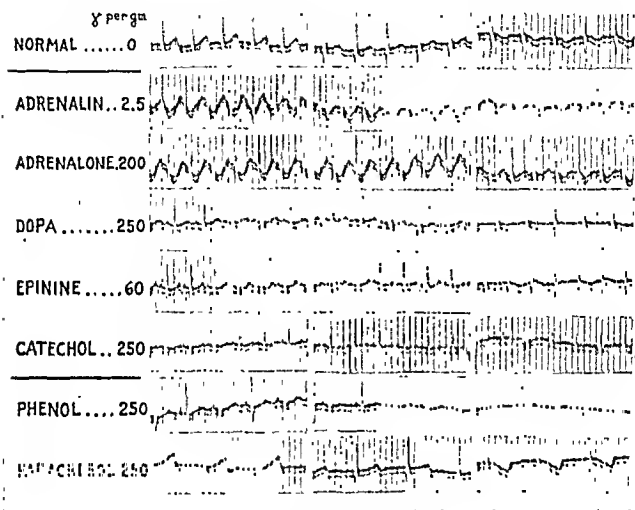


Fig. 13.—Effect of various catechol and phenol compounds upon the electrocardiogram of one rabbit on different days.

For the purpose of comparison with the effects of known catechol compounds, several such substances were injected subcutaneously in large doses into one rabbit (Fig. 13) on different days. In each instance the electrocardiogram was taken fifty minutes after the injection because it was necessary to wait for the disappearance of intense tremors and twitchings which followed some of the injections (particularly of pyrocatechol). Adrenalin and adrenalone produced tachycardia with a depression of the S-T interval and diphasic or inverted T waves. Dihydroxyphenylalanine ("dopa") and epinine (synthetic product) caused some tachycardia and a slight to moderate flattening of the T waves. Pyrocatechol (catechol) also increased the heart rate, reduced the voltage, and caused almost complete flattening or inversion of the T waves (see also Fig. 14 which was made from another animal).

Phenol and paraeresol were tested likewise (Fig. 13). Although the latter caused a marked bradycardia and a change in shape of the T waves, no marked flattening or inversion of the T waves occurred.

EFFECT OF CATECHOL INJECTION (s.c.) (RABBIT)

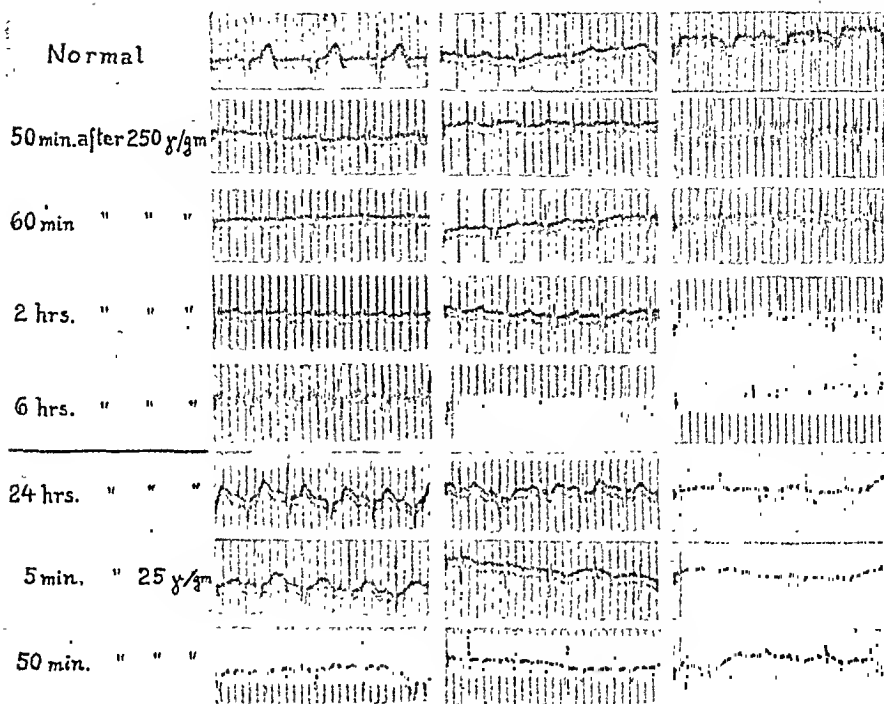


Fig. 14.—Effect of different catechol doses upon the electrocardiogram of a rabbit.

DISCUSSION

Excessive accumulation of catechol compounds (AC) of probably adreno-sympathetic origin (adrenalin and related substances) in the blood as well as in the heart muscle was found to be a characteristic feature of uremia (28 clinical cases).

With only few exceptions the uremic patients displayed signs of cardiac failure and marked abnormalities of the electrocardiogram of the anoxic type.

The uremic sera examined showed specific, intensely cardiotoxic properties as manifested by their effects upon the isolated frog heart and upon the rabbit's electrocardiogram. The latter effects were produced by protein-free serum extracts which contained the entire catechol material and which proved rapidly fatal.

Correlation of the clinical, biochemical, and experimental findings suggested the probable identity of the excess catechol material present in the uremic blood and myocardium with at least part of the cardiotoxic substances present in the uremic serum.

In an attempt to substantiate this conception, model experiments were performed with various known catechol compounds, such as adrenalin, adrenalone, dihydroxyphenylalanine, epinine, and pyrocatechol, on the isolated frog heart and on the rabbit's electrocardiogram. By this procedure some of the

characteristic cardiotoxic effects of the uremic serum could be reproduced: anoxic changes of the electrocardiogram and alterations of cardiac rhythm and contractility, ranging between tachycardia and cardiac standstill and between increase and decrease of cardiac amplitude. All of these partly divergent features are commonly observed in clinical uremia as well.

There is no indication from the chemical findings that adrenalin proper forms a major fraction of the excess catechol compounds accumulated in the uremic organism, but since Richter has shown that adrenalin is normally excreted by the kidneys in a modified form, it appears probable that altered adrenalin and other adrenalin-like substances which are produced by the adrenal medulla and probably by the entire sympathetic nervous system (see Raab,^{1,2} and Cannon and Lissák) are retained in the body in conditions of advanced renal excretory insufficiency.

The general toxic effects of catechol compounds upon the heart muscle are, of course, most strikingly exemplified by adrenalin: stimulation as well as inhibition (Sollmann and Barlow³), myocardial anoxia, structural damage and failure (Raab¹); but in view of the fact that the catechol nucleus is believed to be essentially responsible for the pharmacodynamic effects of adrenalin (Dakin; Tainter; Vaughan, Perkins, and Derbes) and for its high electric potential (Ball and Chen), it appears reasonable to ascribe adrenalin-like cardiotoxic effects also to other biologic catechol compounds as found in excess in the uremic organism.

It must be emphasized, however, that beside catechols, intestinal phenols (bearing only one free hydroxyl group on a benzene ring instead of two) also form a part of the abnormal material which accumulates in the uremic blood. They too, possess certain cardiotoxic properties (bradycardia, decrease of amplitude) and, therefore, must be considered as participating next to the catechols in those chemical mechanisms which bring about cardiac damage and ultimately cardiac death in a large number of uremic patients.

The fatal effect of the accumulation of adrenalin and of other related catechol compounds in the heart muscle above a certain critical level has been discussed elsewhere (Raab¹) on the basis of experimental and clinicopathologic observations.

SUMMARY

Blood and heart muscle of uremic patients were found to contain excessive amounts of catechol compounds of probably adreno-sympathetic origin.

These findings were roughly paralleled by the presence of anoxic electrocardiographic changes and signs of cardiac failure.

The sera of uremic patients displayed specific, strikingly toxic effects on the isolated frog heart and on the heart of the intact rabbit.

Analogous effects were reproduced experimentally by various known catechol compounds and in part also by phenols.

Both catechol and phenol compounds are believed to participate significantly in the chemical mechanism leading to cardiac failure and death in uremia.

REFERENCES

- Agnoli, R., and Bussa, D.: Ricerche cliniche e sperimentali sulle alterazioni elettrocardiografiche esistenti nell' uremia, *Cuore e circolaz.* 23: 2, 1939.
- Ball, E. G., and Chen, T. T.: Studies on Oxidation-Reduction; XX. Epinephrine and Related Compounds, *J. Biol. Chem.* 102: 691, 1933.
- Becher, E.: Studien über die Pathogenese der echten Urämie, *Zentralbl. f. inn. Med.* 46: 369, 1925; *Pathogenese, Symptomatologie und Therapie der Urämie, Ergebn. d. ges. Med.* 18: 51, 1933.
- Brandt, F., and Katz, G.: Ueber den Nachweis von Adrenalinsekretion beim Menschen, *Ztschr. f. klin. Med.* 123: 40, 1933.
- Cannon, W. B., and Lissák, K.: Evidence for Adrenaline in Adrenergic Neurones, *Am. J. Physiol.* 125: 765, 1939.
- Chalier, G., and Contamin, N.: Pulsus Alternans and Uremia, *Progrès méd.* 36: 13, 1921 (Quoted by Gouley).
- Dakin, H. D.: On the Physiological Activity of Substances Indirectly Related to Adrenalin, *Proc. Roy. Soc., London, s.B.* 26: 493, 1905.
- Dickes, R.: Relation Between the Symptoms of Uremia and the Blood Levels of the Phenols, *Arch. Int. Med.* 69: 446, 1942.
- Dosne, Ch.: The Effect of Dosage and Duration of Administration on the Anti-uremic Effect of Desoxycorticosterone, *Am. J. Physiol.* 134: 71, 1941.
- Gouley, B. A.: The Myocardial Degeneration Associated With Uremia in Advanced Hypertensive Disease and Chronic Glomerular Nephritis, *Am. J. M. Sc.* 200: 39, 1940.
- Gross, H., and Sundberg, M.: The Concentration of Creatine in Heart, Diaphragm, and Skeletal Muscle in Uremia, *Ann. Int. Med.* 16: 737, 1942.
- Heitz, J.: Uremia With Pulsus Alternans, *Progrès méd.* 36: 67, 1921 (Quoted by Gouley).
- Kato, M., and Aibara, S.: The Effect of Sympathol and Adrenalone on the Action of Adrenaline, *Jap. J. M. Sc., IV. Pharmacol.* 7, *Proc. Japou. Pharmacol. Soc.* 113, 1933.
- Kendall, E. C.: Hormones of the Adrenal Cortex, *Endocrinology* 30: 853, 1942.
- Küllbs, F.: Krankheiten der Kreislaufsorgane in Mohr-Stähelin's Lehrbuch der Inneren Medizin, ed. 1, (quoted by Agnoli and Bussa).
- Langecker, H.: *Arch. f. exper. Path. u. Pharmacol.* 106: 1, 1925.
- Luisada, A.: The Pathogenesis of Paroxysmal Pulmonary Edema, *Medicine* 19: 475, 1940.
- Lüscher, W.: *Frankfurt. Ztschr. f. Path.* 26: 293, 1921 (Quoted by Gouley).
- Marcolongo, F.: Ricerche cliniche e sperimentali sui fenoli nell' uremia; alterazioni dei fenoli del sangue (volatili, non volatili ed eteroinsolubili) nell' uremia e loro relazione con i fenomeni clinici, *Riv. di pat. sper.* 8: 450, 1937.
- Mason, M. F., Resnik, H., Minot, A. S., Rainey, J., Pilcher, C., and Harrison, T. R.: Mechanism of Experimental Uremia, *Arch. Int. Med.* 60: 312, 1937.
- Merklen and Rabé: V. Congr. de Méd. Française, Lille, 1899 (quoted by Agnoli and Bussa).
- Raab, W.: (1) The Pathogenic Significance of Adrenalin and Related Substances in the Heart Muscle, *Exper. Med. & Surg.* 1: 188, 1943; (2) Corrected Evaluation of the Results Obtained With Shaw's Colorimetric "Adrenalin" Method, *Endocrinology* 32: 226, 1943; (3) Adrenalin and Related Substances in Blood and Tissues, *Biochem. J.* 37: 470, 1943; (4) Unpublished data.
- Richter, D.: The Inactivation of Adrenaline in Vivo in Man, *J. Physiol.* 98: 361, 1940.
- Solomon, C., Roberts, E., and Lisa, J. R.: The Heart in Uremia, *Am. J. Path.* 18: 729, 1942.
- Selye, H.: (1) Beneficial Action of Desoxycorticosterone Acetate in Uremia, *Canad. M. A. J.* 43: 333, 1940; (2) The Effect of Testosterone on the Kidney and on the General Condition of Uremic Animals, *Canad. M. A. J.* 42: 188, 1940.
- Shaw, F. H.: The Estimation of Adrenaline, *Biochem. J.* 32: 19, 1938.
- Sollmann, T., and Barlow, O. W.: (1) The Effect of Epinephrine and Prolonged Accelerator Stimulation on the Response of the Frog Heart to Stimulation of the Cardio-inhibitory Nerve, *J. Pharmacol. & Exper. Therap.* 28: 159, 1926; (2) The Relation of Depressant and Stimulant Actions of Epinephrine on the Frog Heart, *J. Pharmacol. & Exper. Therap.* 29: 233, 1926.
- Tainter, M. L.: Comparative Actions of Sympathomimetic Compounds: Catechol Derivatives, *J. Pharmacol. & Exper. Therap.* 40: 43, 1930.
- Tani, S.: The Toxicity and Cause of Death From the Use of Adrenaline and Adrenalone, *Folia pharmacol. japon.* 13: 393 (Breviaria 28-9), 1932.
- Vaughan, W. T., Perkins, R. M., and Derbes, V. J.: Epinephrine and Ephedrine Analogues and Their Clinical Assay, *J. LAB. & CLIN. MED.* 28: 255, 1942.
- Wood, J. E., Jr., and White, P. D.: The Electrocardiogram in Uremia and Severe Chronic Nephritis With Nitrogen Retention, *Am. J. M. Sc.* 169: 76, 1925.

LABORATORY METHODS

GENERAL

LEUCOCYTOSIS AS AN INDEX OF PYROGENICITY IN FLUIDS FOR INTRAVENOUS USE

B. GORDON YOUNG, PH.D., F.R.S.C., and F. A. H. RICK, B.Sc., HALIFAX, N. S.

SEVERAL tests have been proposed within recent years to detect the presence of pyrogenic substances of bacterial origin in distilled water for intravenous use. Welch, Calvery, McClosky and Price¹ have suggested the measurement of rectal temperature in rabbits with a rise of 0.8°C . or more within three hours as indicative of the presence of pyrogen. They have expressed the potency of their standard preparation of *Pseudomonas aeruginosa* in terms of nitrogen and found that intravenous injections of 0.75 to 1.50 mg. invariably elicited a typical response. The limit was given as 0.83 μg . as calculated by us from their data. Lees and Levy² have also made use of rise in rectal temperature in rabbits as a test for pyrogens.

Co Tui, McClosky, Schrift and Yates³ called attention to the leucopenia in dogs as a criterion of pyrogenicity and accepted a decrease of 5,000 in the white cell count in about forty-five minutes as indicating the presence of pyrogen. Chapman⁴ has studied this method in rabbits and found it about as sensitive as that based on temperature but less stringent as to precautions necessary.

The metabolic products of several organisms appear to be capable of inducing the syndrome of nausea, vomiting, defecation, chills and fever. Bourne and Seibert⁵ associated the phenomenon in varying degree with several non-chromogenic bacteria from river water, Banks⁶ with the chromogenic *Pseudomonas scissa* and *ureae* and Co Tui and Schrift³ with several pathogenic and facultative saprophytic bacteria. The nitrogen equivalent cannot therefore be taken strictly as an index of pyrogenicity.

The phenomenon is apparently so widespread and of such general importance in intravenous medication that a simple test for the presence of pyrogenic substances is very desirable. This is substantiated by a recent collaborative study in 15 laboratories (McClosky *et al.*¹⁰). In the course of an investigation of the alleged leucocytic response to allantoin in man the active agent was demonstrated to be pyrogen (Young and Hawkins¹¹). The leucocytosis appeared to be detectable in the absence of any subjective symptoms and when no leucopenia or hyperpyrexia was demonstrable. These observations have led us to study the relative sensitivity of these criteria for detecting pyrogenic compounds.

EXPERIMENTAL

The plan of the investigation was to prepare a sample of pyrogenic water and to inject this into dogs. Their response was to be noted at successive dilutions in terms of objective symptoms, rectal temperature, and white blood cell and differential counts.

A culture of *Pseudomonas aeruginosa*, obtained from our Department of Bacteriology, was grown on nutrient broth for twenty-four hours at 37° C. This was used to inoculate three Kolle flasks containing Difeo nutrient agar. After incubation at 37° for forty-eight hours, the growth was washed off with 30 c.c. of redistilled water to each flask. The combined washings were agitated for five minutes in a Waring blender, then incubated at 37° for four days. Sodium chloride (Merek's reagent for biological work) was added to make 0.90 per cent and the solution passed through a medium Berkefeld candle. The filtrate was kept sterile at 4° and used directly for injection as the stock standard. This solution was diluted with fresh triply distilled water which had been distilled from (1) a Barnstead still, (2) dilute acid potassium permanganate, (3) dilute barium hydroxide. The last two distillations were made in all-glass stills with fractionating attachments (Pyrex No. 3360 and Fisher No. 9-107). Sodium chloride was added to make 0.9 per cent, and such diluted solutions were immediately passed through the Berkefeld filter. The stock standard contained 5.60 mg. of nitrogen per 100 c.c. determined by the micro-Kjeldahl method in a Pregl apparatus.

Six dogs, varying in weight from 7 to 23 kilograms, have served as experimental subjects. They have been used in preference to rabbits because of the notorious instability of the latter for experimental purposes. The animals were fed Purina dog chow once a day. Fluctuations in rectal temperature and cell counts were observed frequently during at least one day prior to injection with pyrogen. Blood was taken from the ear for white cell and differential counts. For the latter, Wright's stain was used and 300 to 500 cells were identified each time by cross counting with the aid of a mechanical stage. Injections were made into one of the veins in the hind leg. We made use of sodium amytal (Eli Lilly and Co.) as anesthetic initially but discontinued this practice because in some animals it gave rise to a leucocytosis. Observations were frequently necessary over a period of 16 hours to determine the duration of the leucocytosis and consequently it was not accurately determined in a few cases.

In Table I are shown the results of injecting 20 c.c. of tridistilled water and of an old sample of singly distilled water which had been about the laboratory for several weeks. These may be contrasted with progressive dilutions of the stock pyrogenic standard. A typical response was observed with the latter accompanied by a leucopenia of 4,000 cells below the mean and a leucocytosis of 11,000 cells above it. The amount of nitrogen injected was 280 μ g. The leucopenia and hyperpyrexia had disappeared as had any objective symptoms at a dilution of 1:1,000. The leucocytosis was still definite. It was appreciable at 1:5,000 but absent at 1:10,000 based on the reasonable restrictions suggested by Medlar¹¹ that a significant increase should be more than 50 per cent of the

TABLE I
EFFECTS OF PYROGEN ON WHITE BLOOD CELLS AND TEMPERATURE OF THE DOG

DOG	EXPERIMENT	W.B.C. VARIATION	NEUTROPHIL VARIATION	LEUCOPENIA		LEUCOCYTOSIS		TEMPERATURE ° F.	HYPER- PYREXIA ° F.	REMARKS
				MAX.	DURATION	MAX.	DURATION			
A, ♂ 23 kg.	Control	per c.mm. 8,060-8,560	% 60-69	hr.	hr.	hr.	hr.	101.7-102.0	-	20 ml. injected
	Redistilled water	8,240-9,040	63-71	-	-	-	-	101.7-102.0	-	20 ml. injected
	Old distilled water	8,200-12,080	68-74	-	-	-	5	101.3-102.0	-	20 ml. injected
	Pyrogenic water									
	1:0	4,200-19,920	65-92	1	3	7	10	101.6-102.6	0.9	5 ml. injected
	1:10	4,800-19,800	68-90	1	2	9	12	101.6-102.3	0.6	5 ml. injected
	1:1,000	9,010-17,400	65-76	-	-	4	8	101.4-101.5	-	5 ml. injected
	1:5,000	9,000-15,580	67-70	-	-	4	4	101.5-101.7	-	5 ml. injected
	1:10,000	8,200-9,600	68-70	-	-	-	-	101.6-101.7	-	5 ml. injected

original total and 10 per cent of the differential picture. The neutrophilia was not as sensitive an index as the leucocytosis. This would mean that a minimum response was obtained with $0.056 \mu\text{g.}$ nitrogen, equivalent to $0.35 \mu\text{g.}$ pyrogenic protein or $0.015 \mu\text{g./kg.}$ body weight. Weleli, *et al.*,¹³ obtained a minimum response with $0.83 \mu\text{g.}$ nitrogen as indicated by rectal temperature. Seibert,¹² however, has recorded an effect with $0.005 \mu\text{g.}$ pyrogenic protein. It is to be noted that the level of leucocytosis is approximately proportional to the amount of pyrogen in the more dilute preparations.

Fig. 1 presents the observations in detail of a typical experiment done on Dog A as summarized in Table I. It shows a slight effect on the lymphocytes and a typical response to pyrogen by the leucocytes at a dilution of 1:10 of the standard solution.

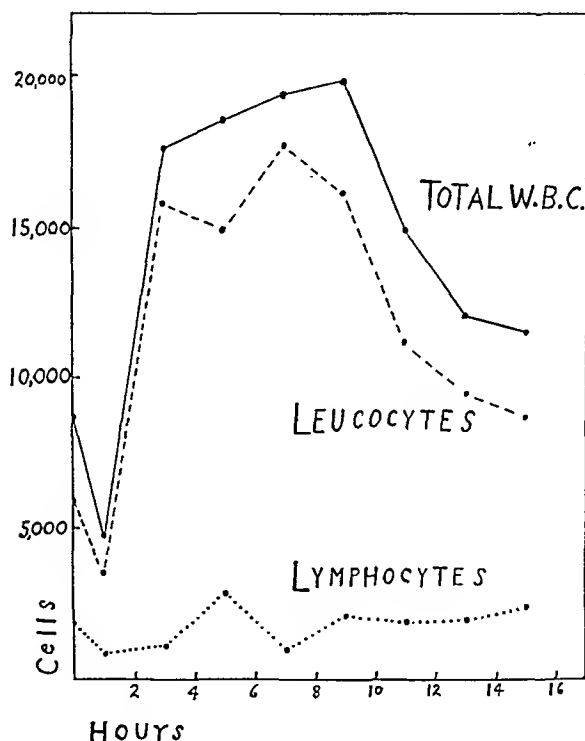


Fig. 1.—Changes in the concentration of the white cell counts after intravenous injection of 5 c.c. of pyrogenic water, diluted 1:10.

Table II presents the evidence from the five dogs used in confirmation of the observations shown in Table I. With the original standard all dogs showed the typical pyrogen syndrome accompanied by a variable hyperpyrexia from 0.5 to 6.1°F. , a leucopenia followed by a leucocytosis and neutrophilia. The leucopenia and hyperpyrexia disappeared first with dilution; the neutrophilia was doubtful at 1:1,000; the leucocytosis became doubtful at 1:5,000 but disappeared at 1:10,000. It is therefore to be concluded that in dogs the leucocytosis is the most sensitive index of the presence of pyrogenic compounds. This confirms the observations made previously in man. (Young and Hawley¹⁴)

TABLE II
EFFECTS OF PYROGEN ON WHITE BLOOD CELLS AND TEMPERATURE OF THE DOG

DOO	EXPERIMENT	W.B.C. VARIATION	NEUTROPHILE VARIATION		LEUCOPENIA		LEUCOCYTOSIS		TEMPERATURE VARIATION ° F.	HYPER- PYREXIA ° F.	REMARKS
			%	MAX.	DURATION	MAX.	DURATION				
B, ♂ 20 kg.	Control	per cmm.	61.69	-	-	-	hr.	101.5-101.9	-	-	5 ml. injected
	1:10	8,240-9,280	34.92	2	3	8	26	101.6-102.2	0.5	-	5 ml. injected
	1:5,000	8,500-12,740	63.70	-	-	3	4	101.7-101.8	-	-	5 ml. injected
	1:10,000	8,320-9,280	64.69	-	-	-	-	101.6-101.7	-	-	5 ml. injected
C, ♀ 7 kg.	Control	8,000-9,300	64.69	-	-	-	-	101.7	-	-	2 ml. injected
	1:0	3,460-19,920	32.83	1	3	8	10	101.6-102.2	0.5	-	2 ml. injected
	1:5,000	8,920-12,020	63.75	-	-	7	4	101.7	-	-	2 ml. injected
	1:10,000	8,760-9,900	65.69	-	-	-	-	101.6-101.7	-	-	2 ml. injected
D, ♀ 7 kg.	Control	8,200-9,000	63.70	-	-	-	-	101.7	-	-	1 ml. injected
	1:0	4,200-16,480	63.52	1	3	8	10	101.6-102.2	0.5	-	1 ml. injected
	1:5,000	8,000-12,220	63.80	-	-	12	3	101.6-101.8	-	-	1 ml. injected
	1:10,000	8,240-8,940	63.70	-	-	-	-	101.6-101.7	-	-	1 ml. injected
E, ♂ 22 kg.	Control	8,100-9,000	63.70	-	-	-	-	101.8	-	-	5 ml. injected
	1:0	2,600-24,140	49.92	2	3	10	12	101.9-108.0	6.1	-	5 ml. injected
	1:1,000	8,670-14,000	65.72	-	-	6	10	101.8-101.9	-	-	5 ml. injected
	1:5,000	8,200-12,020	65.75	-	-	6	7	101.7	-	-	5 ml. injected
F, ♂ 20 kg.	1:10,000	8,520-9,420	66.69	-	-	-	-	101.7	-	-	5 ml. injected
	Control	8,000-9,200	62.69	-	-	-	-	101.7	-	-	5 ml. injected
	1:0	4,080-18,900	40.95	3	4	11	7	101.7-104.2	2.5	-	5 ml. injected
	1:1,000	8,820-18,400	66.75	-	-	8	12	101.7	-	-	5 ml. injected
	1:5,000	8,920-13,200	69.70	-	-	7	4	101.8	-	-	5 ml. injected
	1:10,000	8,320-9,200	68.70	-	-	-	-	101.7	-	-	5 ml. injected

It should be pointed out that there are wide differences as to the normal blood picture of the dog in the literature. Downey⁷ gives the average white blood cell count at 8,000 and the percentage of neutrophils at 60 to 76. Busch and Van Bergen³ found the average white cell count to be 9,526 and the neutrophil variation 54 to 74, avg. 65.7 per cent, on 20 dogs. Mayerson⁹ placed the white blood cell count at 11,165 and the neutrophils at 74 per cent on 60 dogs. Our short series of six dogs showed a normal fluctuation in the white blood cells of 8,000 to 9,300 and the neutrophils of 60 to 71 per cent.

CONCLUSION

By the intravenous injection of small volumes of water or saline (1 to 20 c.c.) into dogs it is possible to detect the presence of pyrogenic substances at or below the level of concentration which will produce symptoms in man. This is determined most readily by the resulting leucocytosis and progressively by the neutrophilia, leucopenia and hyperpyrexia. White blood cell counts alone, however, will suffice to indicate the presence of pyrogens in objectionable concentrations taking an increase of 50 per cent above the original count as significant on the basis of counts made 3 to 6 hours after injection.

SUMMARY

A study has been made on the dog of criteria indicating the presence of pyrogenic substances in distilled water. Leucocytosis was the most sensitive, measurable in three to six hours after intravenous administration and lasting for many hours. Progressively less sensitive were the neutrophilia, leucopenia and hyperpyrexia.

The least amount of solution producing a positive response contained 0.056 μ g. of nitrogen.

The level of leucocytosis was approximately proportional to the amount of pyrogen in the more dilute preparations.

REFERENCES

1. Banks, H. M.: A Study of Hyperpyrexia Reaction Following Intravenous Therapy, *Am. J. Clin. Path.* 4: 260, 1934.
2. Bourne, J. M., and Seibert, F. B.: The Cause of Many Febrile Reactions Following Intravenous Injections, II. The Bacteriology of Twelve Distilled Waters, *Am. J. Physiol.* 71: 652, 1924-25.
3. Busch, F. C., and Van Bergen, C.: Dog's Blood—Differential Counts of Leucocytes, *J. Med. Research* 8: 408, 1902.
4. Chapman, C. J.: Use of Rabbits for Detection of Pyrogenic Substances in Solutions for Intravenous Administration, *Quart. J. Pharm. & Pharmacol.* 15: 361, 1942.
5. Co Tui, McClosky, K. L., Schrift, M. H., and Yates, A. L.: Filtration Studies on Reactive Infusion Fluids, *Proc. Soc. Exper. Biol. & Med.* 35: 297, 1936-37.
6. Co Tui and Schrift, M. H.: Production of Pyrogen by Some Bacteria, *J. Lab. & Clin. Med.* 27: 569, 1942.
7. Downey, H.: *Handbook of Hematology*, New York, 1938, P. B. Hoeber, Vol. 2, p. 825.
8. Lees, J. C., and Levvy, G. A.: Emergency Preparation of Pyrogen-free Water, *Brit. M. J.* 1: 430, 1940.
9. Mayerson, H. S.: The Blood Cytology of Dogs, *Anat. Rec.* 47: 239, 1930.
10. McClosky, W. T., Price, C. W., Van Winkle, Jr., W., Welch, H., and Calvery, H. O.: Results of First U. S. P. Collaborative Study of Pyrogens, *J. Am. Pharm. A.* 32: 69, 1943.

11. Medlar, E. M.: "Rest" and "Activity" Levels of Leukocytes in Health and Disease, *Arch. Int. Med.* 57: 367, 1936.
12. Seibert, F. B.: The Cause of Many Febrile Reactions Following Intravenous Injections. I., *Am. J. Physiol.* 71: 621, 1924-25.
13. Welch, H., Calvery, H. O., McClosky, W. T., and Price, C. W.: Method of Preparation and Test for Bacterial Pyrogen, *J. Am. Pharm. A.* 32: 65, 1943.
14. Young, E. G., and Hawkins, W. W.: The Role of Pyrogens in the Alleged Leukocytic Response to Allantoin, *J. Pharmacol. & Exper. Therap.*, in press.

ON THE MOBILIZATION OF IRON IN HEMOCHROMATOSIS WITH ADMINISTRATION OF VARIOUS CHEMICALS*

HAMILTON R. FISHBACK, Sc.D., M.D., CHICAGO, ILL.

DIFFUSE iron deposits in tissues of the body are pathologic. If in sufficient amount, the presence of iron may be associated with abnormal changes of the organ parenchyma. Atrophy and fibrosis may occur as in the liver and pancreas with hemochromatosis. Chronic inflammatory change may be present in Kasehin-Beck's disease of the joints. The problem of mobilizing abnormal stores of iron and discharging it from the body appears to have had very little attention. This contrasts quite markedly with the efforts put forth toward finding the means of eliminating other metals which are detrimental to the tissues in which they are stored or to the whole body.

In this work some of the agents ordinarily used for the removal of heavy metals from the body were administered, and their effect on the iron balance was measured. The subject was a white man admitted to the hospital with a diagnosis of hemochromatosis in a late stage.

Previously he had been getting along quite well under the care of his family physician. However, while arrangements for metabolic study were being made, his abdomen suddenly became distended and his temperature rose to 105° F. rectally. On instituting stomach drainage he improved rapidly so that by the next day his abdomen was soft and the temperature was 99° F. The liver was quite enlarged and there was some ascites. The diabetes was well controlled with insulin.

After 16 days of medical management he was put on a standard diet of known iron content, with daily collection of urine and stools for chemical analysis. Period separation of feces was made with carmine and charcoal. After a control period of 4 days he was given the following compounds in order, each for a period of 7 days: Sodium thiosulfate, 8½ Gm. daily, divided into three intravenous doses; ammonium chloride, 6 Gm. daily, given orally in three doses; sodium bicarbonate, 9 Gm. daily, in three oral doses; sodium thiosulfate, 17.5 Gm. daily, given intravenously in three doses. The iron intake was kept at a very low level.

Table I shows the details of iron intake and excretion.

Results.—In the control period the excretion of iron in the feces was increased by blood which was found later by chemical tests to be present in the feces of the first two days. Unfortunately it was not feasible to repeat the control period. Blood was not found in the stool in any other period. In Period II there was an appreciable iron retention. The question must be considered, however, of a retention possibly to equalize the iron loss of the preceding period.

*From the Department of Pathology, Northwestern University Medical School.
Received for publication, Feb. 19, 1944.

TABLE I

PERIOD		TIME	MG. OF IRON INTAKE		MO. OF IRON OUTPUT		MG. OF IRON DAILY EXCRETION		IRON BALANCE DAILY AS MG.
			TOTAL	DAILY	TOTAL	DAILY	URINE	STOOL	
I	Control	4 days	21.24	5.31	48.27	12.07	2.31	9.76	-6.76
II	Sodium Thiosulfate 8½ Gm. daily	7 days	38.0	5.40	21.60	3.08	0.69	2.39	+2.32
III	Ammonium Chloride 6 Gm. daily	7 days	38.0	5.40	31.29	4.47	1.81	2.66	+0.93
IV	Sodium Bicarbonate 9 Gm. daily	7 days	41.4	5.91	40.27	5.75	2.49	3.26	+0.16
V	Sodium Thiosulfate 17½ Gm. daily	7 days	41.4	5.91	64.40	9.23	2.42	6.81	-3.32

In Period III, with the urine made quite acid, there was a very small iron storage. However, the urine was made alkaline in Period IV and again there was slight iron retention. It was decided to push up the thiosulfate dosage to a very high level in Period V. This was done safely, with no evidence of any unfavorable reaction. There was quite an appreciable iron loss with the greater proportion found in the feces. As stated before, blood was not found in the stool in this period.

The urinary excretion of iron in most of the periods was a fairly constant amount. It is sometimes stated to be insignificant as regards total excretion of iron. However, it can be seen in the table that if the figures for urinary iron of the Periods II and V could be exchanged arbitrarily, the significant balances of those periods would be transformed to rather weak ones.

Discussion.—The method of ridding the body of a stored metal may depend not only on the particular metal, but also on the structure in which it is stored. Thus, since lead is deposited in bone along with calcium, it is taken out again by methods which mobilize calcium. Silver deposited in the skin is removed with great difficulty by local treatment because of its insolubility. Bismuth is stored in high amounts in the liver and kidneys and appears to be excreted directly from those organs. Among other metals sometimes found in excess in the body tissues are: mercury, arsenic, zinc, copper, and chromium. There is no known method of accelerating the excretion of any of the above metals by increasing the activity of the liver, kidneys, intestines, or skin.

The direct solution of a metal stored in the body tissues by a chemical given by mouth or intravenously would appear to be difficult. However, this may be accomplished indirectly if the chemical administered reacts with the stored metal to form a soluble salt which is then excreted. This was formerly thought to be the method of action of both potassium iodide and sodium thiosulfate.

It is possible also that salts which supply an excess of available acid or alkaline radicals in the body fluids provide a medium for readier solution of stored metals. This seems to be true of the use of ammonium chloride to mobilize calcium and with it other metals stored in bone.

Iron is often stored as the difficultly soluble hemosiderin. Yet, after a contusion, hemosiderin may be piled up in the tissues in considerable amounts,

but is dissolved and removed within a limited number of days. The iron stores of Addisonian anemia can be taken up and used for building hemoglobin with remission of the disease.

Whether the iron deposited in hemochromatosis can be resorbed either for excretion or hemoglobin formation is not yet known. Even the form in which the iron is stored is not fully understood. Since, however, the sites of greatest iron deposit are subject to profound functional and anatomic change, it would appear important to prevent its deposit or remove it early.

In this work the significance of the negative iron balance of the control period with loss of blood from the intestine could be discussed only if a longer period of loss of a known amount of blood could be correlated with the new formation of hemoglobin from absorbed stores of iron in the tissues. It may be that blood loss furnishes the only feasible method of ensuring a negative balance of iron. Work on this part of the problem is under way.

Summary.—Various chemicals were given to a man with hemochromatosis, over periods of seven days each, with the purpose of attempting to mobilize stored iron. The intake of iron was kept to a known low level, and the output of urinary and fecal iron was determined.

The results do not indicate any striking effect on iron balance by the chemicals given, namely, sodium thiosulfate, ammonium chloride, and sodium bicarbonate.

Sodium thiosulfate in large daily dosage caused a low negative iron balance, but this was outweighed greatly by the iron loss of the control period due to escape of blood in the stool.

Acknowledgment is made here to the valuable support given this work by the Davella Mills Cancer Foundation Fund of the Wesley Memorial Hospital.

A METHOD OF OBTAINING COMPRESSED AIR FOR THE SMALL LABORATORY*

BEECHER L. SCUTCHFIELD, A.B., M.T., WHEELWRIGHT, KY.

COMPRESSED air is a necessity in securing a hot flame for pulling or blowing glassware, drying pipettes or other glassware, and cleaning objects of dust particles. Obtaining compressed air for these purposes presents a problem to most workers in small laboratories. Since compression equipment is too expensive for such laboratories, the author has adapted a vacuum cleaner with a few added attachments.

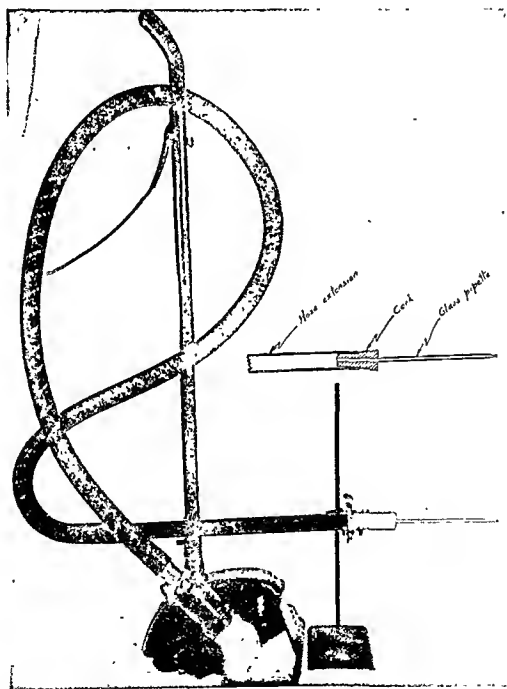


Fig. 1.—Side view of the completed compressor and a detailed drawing of the outlet.

*From the Inland Steel Company Hospital.
Received for publication, Jan. 19, 1944.

The hose of the blower part of the vacuum cleaner is attached to a *small outlet*, thereby producing a *steady* jet of compressed air. The equipment includes an electric vacuum cleaner with a hose attachment, a cork $1\frac{1}{2}$ inches in diameter, $\frac{3}{8}$ inch glass tubing or a 10 c.c. measuring pipette, and a small amount of adhesive tape. The vacuum sweeper must have a blower outlet and hose extension.

The dirt bag of the vacuum cleaner is removed and the hose is attached to the blower outlet. A hole is bored in the cork, into which the glass tubing is fitted. The cork is placed in the exit end of the hose attachment. The exit end of the glass tubing may be small or large. A tube with a small opening is used to produce a small jet of air which may be directed into the flame of a Bunsen burner to produce a blow torch. A 10 c.c. pipette cut and broken at the 2 c.c. mark may be fitted into the cork instead of the glass tube. If the air is to be used for drying glassware or pipettes, a large opening is provided with $\frac{3}{8}$ inch glass tubing and a length of rubber tubing. This may be introduced into flasks or attached to pipettes. To obtain an airtight connection, adhesive tape is placed around the end of the hose extension and the adjoining cork. In addition, two pieces of adhesive tape about 1 inch wide and 9 inches in length are placed at right angles to each other on these connections. To do this, a hole is made about midway of one tape which is then slipped onto the tubing. The tape is attached to the cork and to the extension tube of the sweeper, and bound down with the second tape.

An apparatus support stand and a universal extension clamp will hold the hose outlet steady. To prevent the sweeper from picking up dust particles, it is placed on a large cardboard or on a clean floor. The compressor may be turned on and off with the sweeper switch. Fig. 1 shows a side view of the completed compressor and a detailed drawing of the outlet.

A SIMPLE AUTOMATIC PIPETTE

FIRST LT. HARRY A. KORNBERG, SN. C., A. U. S.

THE shortage of man power in many clinical laboratories has made desirable the application of labor-saving devices to routine procedures. In this communication is described such a device which is being used successfully in the author's serology laboratory for the automatic pipetting of colloidal gold.

Although its application has been limited to the rapid delivery of 2.5 c.c. portions, variations in the dimensions of the apparatus will result in the delivery of larger or smaller amounts, extending its use to other procedures in serology as well as to bacteriology and chemistry.

It will be noted that the solution being pipetted contacts no rubber, cork, or other material that may affect the reagent. This is of special importance when colloidal gold is used in the automatic pipette.

Although other designs utilizing the same principle may be used, the one described here was chosen because of its simplicity and the ready availability of material for its assemblage. The colloidal gold solution is contained in a three liter flask fitted with a 2-hole rubber stopper through which passes the delivery tube, bent to the shape illustrated in (2) Fig. 1. Since the flask must be two to three feet above the workbench, the descending arm of the delivery tube is approximately twenty inches long. The end of the tube is fitted onto a stopcock (3) flanged at one end, such as part of a Van Slyke carbon dioxide apparatus, which, in turn, leads into a Kahn tube (4) supported by a clamp attached to a ring stand. Over the side of the Kahn tube is laid the small siphon tube (5) prepared as in Fig. 1. Cleaner cuts of aliquots are obtained if the lower end of the siphon tube and the tip of the funnel are heated, dipped into molten paraffin, and the excess is shaken off to give a thin film of wax.

To operate the pipette, the stopcock is opened, air is forced through the short length of tubing fitted into the flask's stopper, and the stopcock is adjusted to a convenient rate of flow. Capillary attraction will allow the siphon tube to operate just before the level of the solution reaches the top of the Kahn tube. Since the rate of flow through the siphon tube must exceed that through the delivery tube, the level of the liquid in the Kahn tube will fall until it reaches the end of the siphon tube when the flow through it will cease. When the liquid again reaches its former height, the siphon will operate as before. Thus, uniform amounts of solution are automatically delivered into the funnel through which they may be directed into the test tubes.

In using the apparatus to pipette many aliquots, we have found it convenient to shove the test tube rack along with one hand, the other moving the funnel suspended by a rubber band down into the mouths of the tubes consecutively as the portions are delivered.

Although it has been found as accurate (0.1 c.c.) as 10 c.c. Mohr measuring pipettes, it is well to check the volumes delivered and at the same time provide

practice in its use with tap water in the flask. The shorter arm of the siphon tube may have to be lengthened or shortened, depending on whether the quantities expelled are too little or too much.

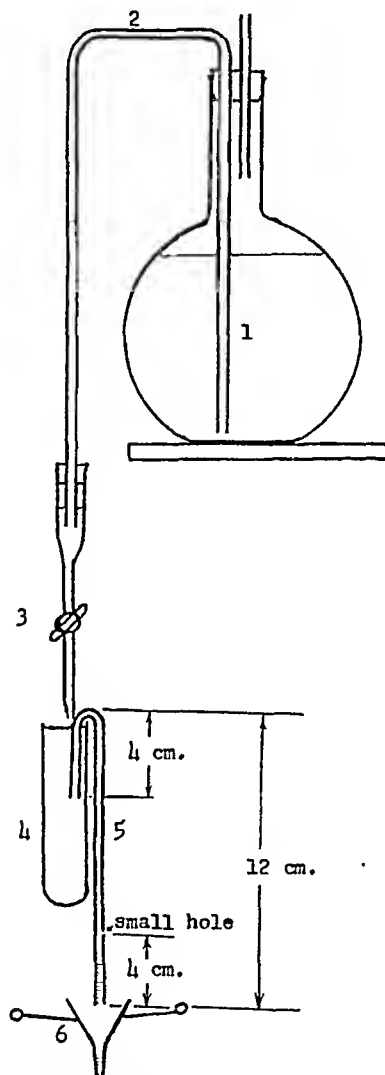


Fig. 1.—1, 3 L. flask; 2, 4 mm. delivery tube; 3, stopcock; 4, Kahn tube; 5, 4 mm. siphon tube; 6, 2-inch funnel suspended on rubber band from iron ring.

The smooth operation of the pipette depends partially upon surface tension. Hence, the upper part of the siphon tube should be cleaned thoroughly by dipping it into strong cleaning solution and rinsing in tap and distilled waters just before using. A useful refinement is to seal another stopcock in series with the one already present. Thus, one will serve to turn off and on the flow of the solution completely without disturbing the other which is adjusted to the proper rate of flow.

I wish to express appreciation to Miss Sarah G. Miller, Major John T. Cuttino, and Sergeant James Mori for many helpful suggestions.

A SIMPLE APPARATUS FOR PERIODIC TESTING OF *P. NOTATUM* FLASK CULTURES

FANCHON HART, F.D.A., B.S., M.A., AND BERNARD L. BLUMBERG, B.S., PHAR.D.,
NEW YORK, N. Y.

RECENTLY the authors were interested in obtaining small quantities of penicillin broths for a research problem. Production of penicillin was carried out in Erlenmeyer and Fernbach flasks by growing *P. notatum* in surface culture on yeast extract-carbohydrate-mineral salt medium. During these experiments potency tests were made at intervals in order to determine the most appropriate time for harvesting the broth. At first the quantity required for potency test was removed by pipette, but since this necessitated some handling of the flask with consequent disturbance of the culture, the apparatus diagrammed below was devised. This setup is easily constructed from ordinary laboratory equipment, and should be useful generally for investigations on metabolism broths which are to be tested periodically for antihacterial potency and perhaps other properties.

EXPERIMENTAL

The siphons were assembled and attached to 750 ml. Erlenmeyer or 2,800 ml. Fernbach flasks. One hundred or 150 ml. of medium was added to the Erlenmeyer, and 400 or 600 ml. to the Fernbach flasks. Cotton plugs were inserted in the mouths of the flasks and the tops were covered with paper in the usual manner. After the siphon had been started by applying suction to the mouthpiece, the pinchclamp was closed, and the protective sleeve was slipped around the mouthpiece. The entire unit was then autoclaved at 121° C. for twenty minutes. The siphon remained filled after autoclaving, and liquid could be withdrawn from the flask at any time by releasing the pinchclamp.

The sterile medium was seeded by pipette with an aqueous spore suspension of *P. notatum*. Cultures were incubated at 23-24° C. When a quantity of metabolism broth was required for potency testing, a sterile tube, outside diameter 9 mm., previously marked at the 4 ml. level was inserted up to the mouthpiece, and 4 ml. of liquid was withdrawn. This volume which represented about 20% more than the predetermined total volume of the siphon was regarded as the contents of the siphon and was set aside. An additional 2 ml. was then drawn off into a second sterile marked tube for potency testing. In this manner the broth in the siphon from the previous test was removed each time before the quantity for potency testing was taken.

Using routine aseptic precautions, this procedure was repeated eight times over a period of sixteen days on some of the flasks. No obstruction of the

siphon or contamination of the culture was noted in any instance. The morphological characteristics and rate of growth of the culture appeared the same in flasks with and without the siphon attachment.

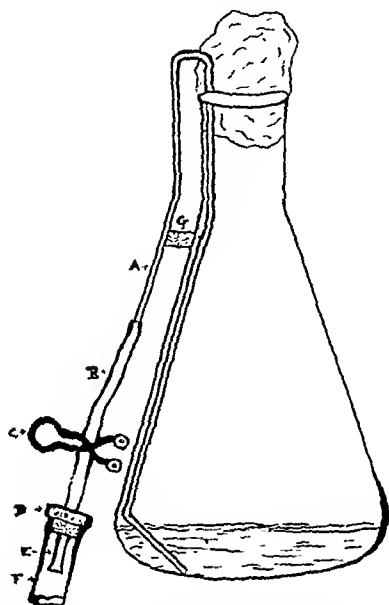


Fig. 1.—Apparatus. *A*, glass tubing, outside diameter 4 mm., wall thickness 1 mm., total length 15 inches (9 inches inside flask, and 6 inches outside); *B*, blood pipette rubber tubing, length 8 inches; *C*, pinchlamp; *D*, rubber tubing, outside diameter 12 mm., wall thickness 3 mm., length 8 mm. Protective sleeve secured on this; *E*, blood pipette mouthpiece; *F*, protective sleeve; glass tubing, inside diameter 11 mm., length 35 mm.; *G*, No. 1 cork stopper as wedge to hold siphon stationary.

SUMMARY

The removal of small quantities of metabolism broth from culture flasks with little disturbance of the culture can be accomplished by the technique and apparatus discussed herein. It seems reasonable to assume that this method might be applied to studies on other fungi which grow similarly where it is desired to test not only the potency of the broth but also changes in pH. By assembling three or four siphons on a single flask, it should be possible to follow pH and potency periodically at various locations and levels in the medium.

MAINTENANCE OF MOISTURE IN BACTERIOLOGIC CULTURE MEDIA

GEORGE H. CHAPMAN, NEW YORK, N. Y.

MANY laboratories prepare culture media and store them for future use but they do not seem to have appreciated the effect of partial dehydration caused by evaporation of moisture from the surface. For example, we have often received cultures on slants of Loeffler's blood serum medium or on blood agar that had become excessively dry. Many microorganisms thrive best in the presence of abundant moisture. Also the concentration of ingredients is affected by dehydration. Hence it is essential to minimize losses of moisture.

In this laboratory, Petri dishes with porcelain covers glazed on the outside are poured approximately 25 ml. each and are stored in an electric refrigerator. The average loss from evaporation, judged by loss of weight, is 0.10 ml. per day. Consequently, the date each medium is poured is noted on a slip of paper which is attached to the set of dishes. When a plate is needed sterile water is added, 0.10 ml. for each day since it was poured, and is spread lightly by a sterile glass spreader. The plate is allowed to stand about a half-hour and is then inverted to drain off any unabsorbed moisture. It is then ready for use.

We have kept different types of blood agar and media containing critical concentrations of bacteriostatic agents for considerable periods of time and have found them entirely satisfactory while other plates from the same lot but not so treated gave poor results.

A VIEWING DEVICE FOR READING KAHN REACTIONS

FLOYD SELL, B.S., DETROIT, MICH.

A VIEWING device is herewith presented which permits the reading of Kahn results without the need of removing the tubes from the Kahn rack. It also permits the reading of individual tubes, when necessary.

The device is so constructed as to permit the transmission of light originating from a daylight fluorescent lamp in such a manner that any precipitate present in the test tubes is illuminated and readily observed in contrast with the clear liquid in which the precipitate is suspended. The device contains shields for the purpose of excluding all extraneous light from the observer's eye. The standard Kahn rack is attached to a tilting lid, thus making possible fine adjustments of the position of the rack, to suit individual workers.

DESCRIPTION OF THE VIEWING DEVICE

The device is enclosed in a hardwood box, measuring approximately 19 × 5 × 6 inches, thus requiring a minimum of space. The box opens with a lid which carries on its underside the holder of the standard Kahn rack. The lid can be fixed by means of a screw at an angle best suitable to the reader. Space is provided at either side of the rack holder where single tubes can be viewed for more detailed study after having been lifted from the rack. A 15 watt daylight fluorescent lamp with its auxiliaries is attached to the bottom of the box (Fig. 1). The underside of the lid is painted flat grey and the inside cavity of the box, black, to provide a dark background; a flat black baffle is used to screen out all excess light. The bottom of the box is slightly curved in order to permit some motion of the box and corresponding motion of the fluid in the tubes as it is observed for the presence of precipitates. A diagrammatic plan of the viewing box is presented in Fig. 2.

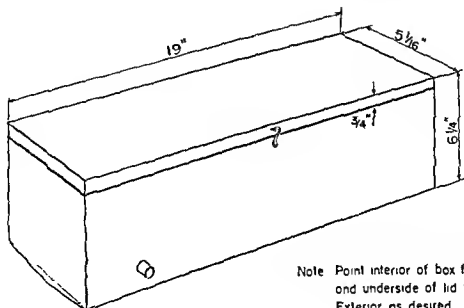
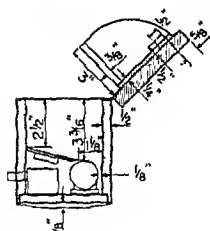
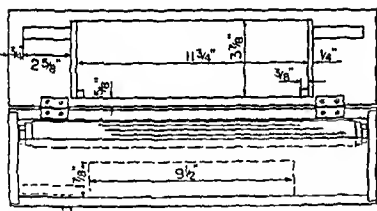
PROCEDURE OF READING

The rack with tubes containing the completed Kahn test is placed in the rack holder (on the underside of the lid) and the lid is fixed at such an angle that the tubes are almost horizontal to permit observation of a thin layer of solution. The observer directs his eyes at the tubes at such an angle as to receive as little extraneous light as possible (Fig. 3). The tipping of the box causes the fluid in the tubes to undergo slight motion thereby permitting better distinction between precipitates and scratches which may be present on the walls of the tubes. If desired, a single tube may be taken out of the rack and viewed

From the Laboratories of the Detroit Edison Company.
Received for publication, Feb. 23, 1944.



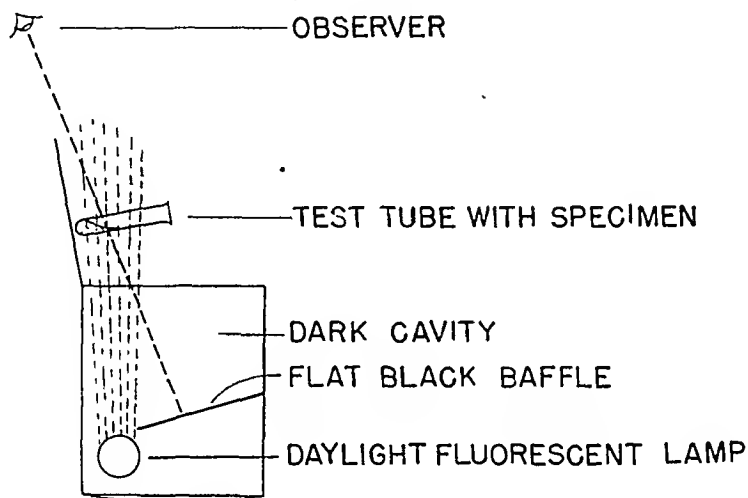
Fig. 1.



Note Point interior of box flat black
and underside of lid flat grey
Exterior as desired

Fig. 2.

at either sides of the rack in a horizontal position parallel to the axis of the lamp. All readings on this device should be made preferably in a darkened room.



SCHEMATIC SECTION

Fig. 3.

SUMMARY

A viewing device for the Kahn test is described which makes possible the reading of precipitation results of an entire Kahn rack or of individual tubes with relative ease.

The author wishes to thank Dr. John Kasper of the Detroit Health Department Laboratories and Dr. R. L. Kahn of the University of Michigan, Ann Arbor, for assistance in this work.

A KAHN VIEWING DEVICE WITH STANDARD ILLUMINATION FOR READING TUBES IN THE RACK*

MAJOR REUBEN CARES, M.C.

THE need for standardizing reading of three-tube flocculation types of serology tests is especially marked when the light conditions can, and do, fluctuate. Many, if not most, laboratories read results by window light. Several textbooks^{1, 2, 3} describe how window or other light sources can be adjusted so that some standard or uniform illumination for reading serologic test tubes will result. Obviously, the time of year, or day, the presence or absence of clouds or sunlight will all affect not only the quantity of light but even the nature of light on the tubes. All other conditions being equal with regard to the technique, the marked range in light between a gray overcast winter sky and the brilliant blue summer sky can affect the accuracy of the individual worker in interpreting degrees of precipitation in these test tubes, particularly in borderline positive-negative zones.

The reading devices now available on the market do feature constant illumination for the tubes, but they require removal of the tubes of each test from the standard Kahn rack to the instruments. Where large runs of flocculation tests are performed, most workers choose the easier and more rapid method of reading the entire rack of 10 tests in either window light or in some convenient lamp light. Homemade devices using a uniformly illuminated light-box background are, of course, a substitute for varying daylight source. The usual disadvantage of such an arrangement is reading of the tube suspensions against a bright background. Generally all, or a great part of a rack, is viewed at one time. Eyestrain may cause lower reading efficiency. Since the brightness range between such a light source, and the general laboratory illumination may be too great, the resultant strain may, of course, lower accuracy in reading finer precipitates. Similar strains have been studied by Lowry⁴ in connection with viewing motion pictures. A brief discussion of eyestrain factors in laboratory tests requiring fine observation will be found below.

FEATURES OF KAHN RACK VIEWING DEVICE (SEE FIGURES)

The device presented (a) eliminates a variable light source, (b) introduces the feature of side lighting against a dark or black background (admittedly the most critical type of illumination), and (c) allows handling of the tubes without removal from the racks used throughout the procedure. It can easily be constructed out of simple materials and is applicable to any test of the flocculation type requiring the standard Kahn rack. As will be noted in Fig. 1, the light source employs oblique lighting with reference to the optical axis of the laboratory worker. The angle of 13° from the perpendicular is that of maximum

*From the Laboratory, Station Hospital, Camp Cooke, California.
Received for publication, Mar. 14, 1944.

acuity with reference to any three-tube test in the rack. The first or last of the tests in the rack can be read under the same light conditions as any intermediate test. It will be noted that this particular angle places the back tube, with respect to the light source, exactly between the other two tubes based on projection lines parallel to the axis of the light source. Similarly the front tube with respect to the viewer's line of vision occupies the midpoint between the other two tubes (middle and back tubes) based on projection lines parallel to the optical axis.

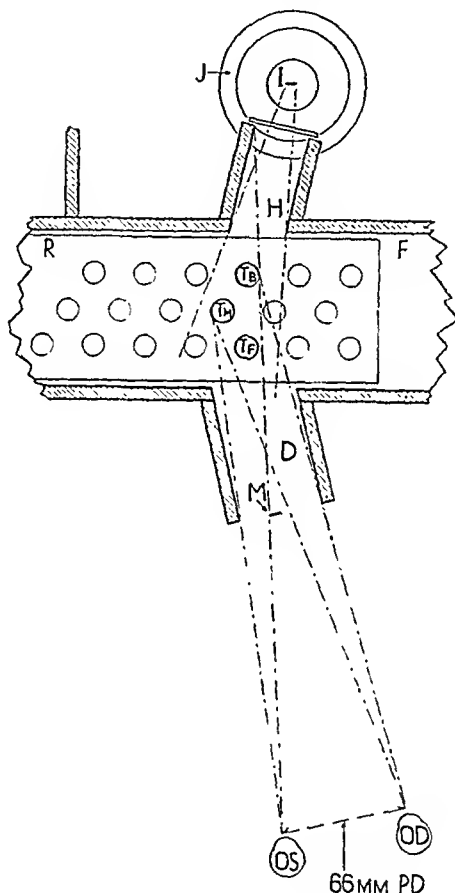


Fig. 1.—Optical features of Kahn viewer.

- A—Panel of viewing tube.
- B—Front panel of lamp house.
- C—Back panel of lamp house.
- D—Viewing tube.
- E—Adjustable lamp base.
- F—Floor of trough for Kahn rack.
- G—Lamp housing.
- H—Tube for light beam.
- I—Standard projection lamp—T8 ½, 100 w., 115 v.

- J—Clear blue glass filter.
- K—Set screw for adjustment and centering of lamp filament.
- L—Lamp housing vents.
- M—Eye shield for view alignment.
- R—Standard Kahn rack.
- Tf—Front serologic tube (lowest dilution).
- Tm—Middle tube.
- Tb—Back tube.

Since only one test with its three tubes is read at a time, the field of view of the rack is best confined, as much as is practicable, to the three tubes of this particularly numbered test. Moreover, the tubes examined received the maximum amount of light and so allow visual disregard for the tubes of adjacent tests in the rack.

While the light source, which is fixed, will form a *field of illumination* of constant dimensions, the same does not hold for the *field of view* in the viewing tube. Obviously, with a fixed diameter aperture for the viewing tube, the nearer the eyes the greater is the angle of vision and the more tubes would be visible. The dimensions of the viewing tube, which is adapted for unaided binocular vision, were determined by the average eye-to-object reading distance of 10-12 inches and an average interpupillary distance of 66 mm. Such variations as exist in reading distance or pupillary distance among serological technicians may be readily and almost unconsciously adjusted for by changing eye-to-object distance.

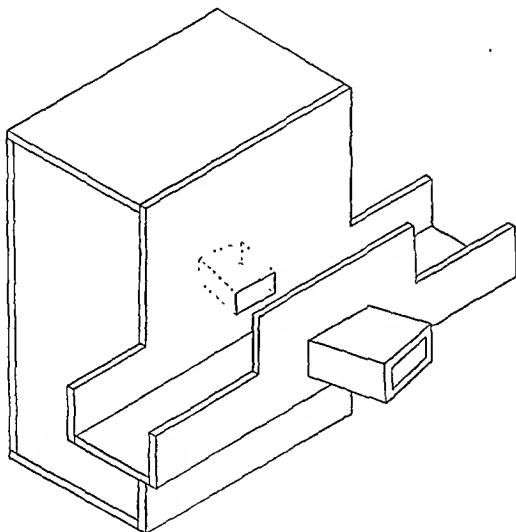


Fig. 2.—Isometric perspective of Kahn viewer.

Fig. 2 is a perspective of the viewing device. This consists essentially of a trough through which the Kahn rack may be slid, a viewing tube, and a light source in an appropriate lamp house with illumination directed through a light tube at a fixed oblique angle.

It will be noted in the front view of the device (Fig. 3) that the panel *A* of the viewing tube reaches only to the top level of the Kahn rack. This is so that all the tubes can be surveyed at a glance for ready reference. The side cut-outs of both the viewing tube panel and the front panel of the lamp house will allow ready grasp of the rack at either end for shaking or other manipulation. In this way almost as much freedom in handling and reading of the ten three-tube tests in a rack is afforded as would be the case in reading by the usual window light.

A few minor refinements are incorporated in this device which require slightly extra equipment or added construction details. The blue glass filter *J* tends to give sharper definition of fine precipitates than straight Mazda light. The lamp block *E* with a set screw *K* allows ready adjustment and centering of

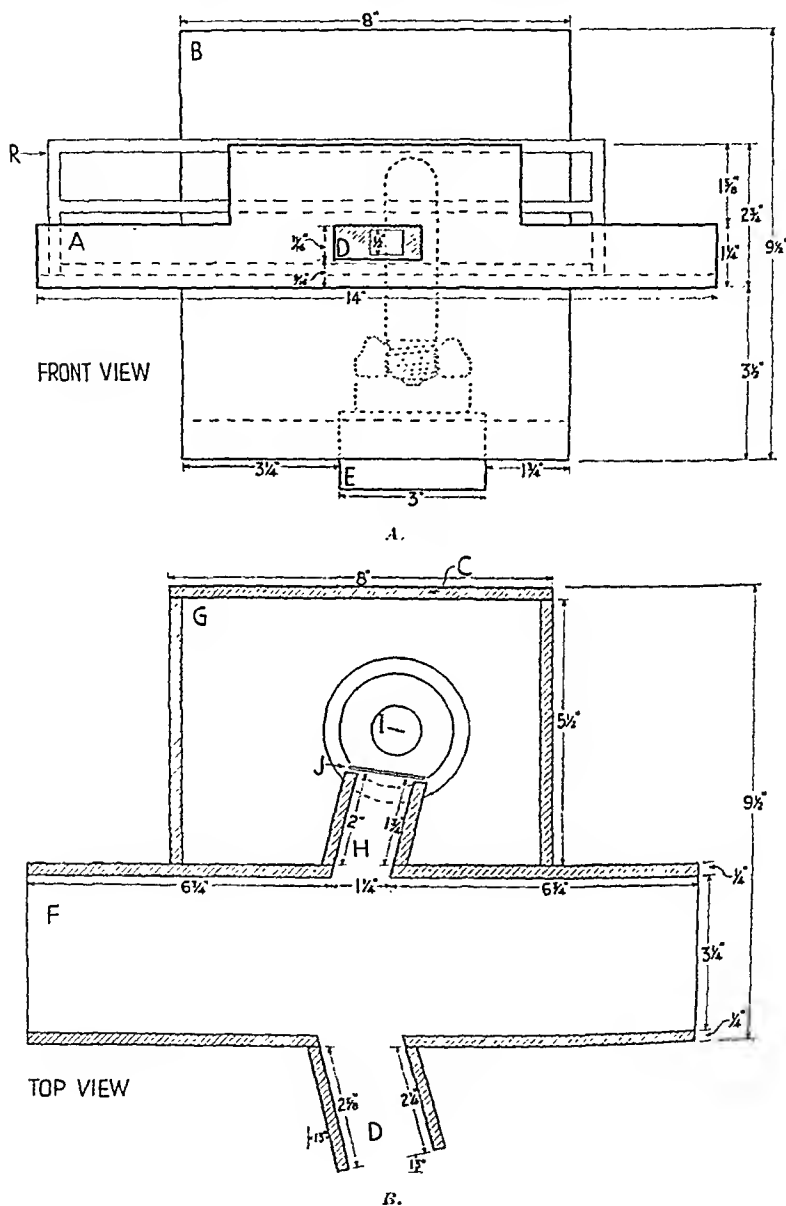


Fig. 3.—Various aspects of device.

the point source of illumination of the concentrated projection filament bulb employed. The lamp housing, being constructed of 1/4" plywood, must be of necessity somewhat more spacious than a metal housing. The latter could be constructed at somewhat greater cost of time and labor.

The table for housing the device (Fig. 4) is designed to be placed on the laboratory bench of 42" average height. When the viewer is placed into the appropriate cut-out in the table top, the tubes in the rack will be at the average eye level used in reading flocculation tests. The height and outside dimensions of the table may be altered to suit the individual laboratory. The table eliminates the necessity for holding the racks and frees both hands for recording of tests or for related procedures.

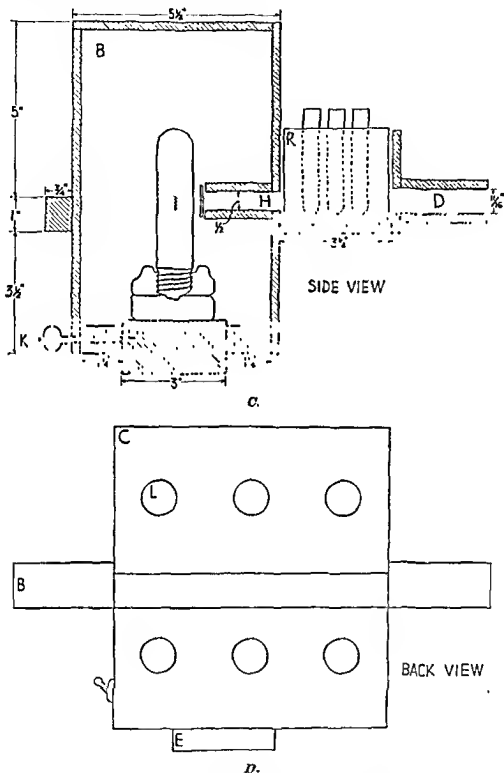


Fig. 3 Cont'd.—For legend see opposite page.

SUGGESTED REFINEMENTS AND IMPROVEMENTS FOR VIEWER

Basically the device consists of a directional trough for sliding the Kahn rack into the field of view of a peep tube. A number of refinements for increasing the usefulness of this device are offered. These fall roughly into 3 groups—optical, mechanical, and those related to constructional material.

a. Suggestions for Optical Improvements.

1. A magnifying lens of 2-4 power may be incorporated in the body, end, or base of the viewing tube. A slot for ready removal or insertion of such a lens can be made.

2. The viewing tube, of rectangular or cylindrical form, may be in telescoping sections for ready adjustment by the individual worker. A more complicated binocular tube arrangement can be constructed which should, of course, readily adjust for various pupillary distances.

3. The aperture at the base of the viewing tube, i.e., nearest the Kahn rack, could be widened by a sliding panel to include several sets of tubes for comparison of tests.

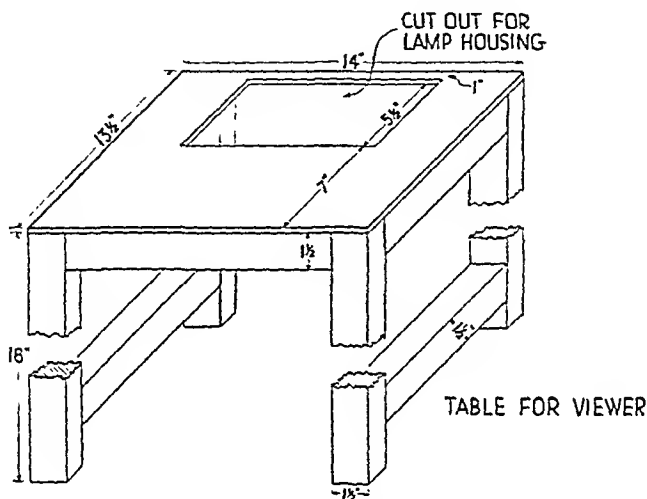


Fig. 4.—Isometric perspective of table for Kahn viewer.

4. The entire device could be further simplified by eliminating the lamp housing *G* and substituting a standard microscope lamp of sufficient light strength. Naturally the angle of the incident light on tubes should be as near as possible to 13° from the perpendicular with reference to the long axis of the trough (and the rack). Otherwise the glare from a more oppositely placed back light may cause visual discomfort to the examiner.

5. A simple frosted glass bulb may be substituted for a projection bulb as a light. However, to prevent the glare from this broader light source the length of the tube for the light beam would have to be increased. This is to insure that the reader does not see the lamp in the field of view of the test tubes. Naturally the longer the light beam tube the less possible will it be to catch a glimpse of the light source through the viewing tube due to the diverging angles (see Fig. 1). The intensity of incident light will fall off if a long light tube is used, so increased wattage may be needed.

6. A precision optical set-up with lamp mirror, designed along film projector lines may be used. It can be incorporated in the lamp housing.

b. Mechanical Refinements.

1. An arrangement for tilting the level floor of the trough for the rack (*F* in Fig. 3*B*) for reshaking tube precipitates could be used.

2. The view tube and tube for the light beam may be made of tubular material with rectangular masks inserted at the tube ends. Since the position of the apertures of both the light and viewing tubes are designed to illuminate only those portions of the three tubes which contain the solutions, reflection or glare from Kahn tube glass above the level of the fluid is minimized. Such rectangular masks of various sizes can be easily constructed so as to slip on readily over the end of the circular or rectangular eye tube.

3. The light shield *M*, as shown in Fig. 1, can be made adjustable to exclude direct rays of the light source from the field of view. It will be noted that the line of vision of the left eye of the observer will permit some direct light glare into this eye. The light shield when in place will not interfere with binocular vision.

c. Materials of Construction.

1. Since the mechanical strength of the viewer requires only sufficient sturdiness to sustain the weight and manipulation of one loaded Kahn rack, alternative materials may be used. All inside dimensions being maintained, some reduction in the bulkiness of the viewer plus its sustaining table can be effected by utilizing sheet metal. Substitutes for plywood may be used.

2. It is even feasible in some laboratories to build-in the apparatus into the wall above the serology workbench so that only a trough and the eye-tube project from the wall above the workbench. An artificial window effect is thus obtained.

3. For those workers who prefer the area surrounding the reading device to be fairly dark, a broad panel, either fixed or removable, can be attached to the back of the lamp housing so no extraneous back lighting would interfere with reading. This would apply, of course, particularly when the apparatus must be placed near or in front of a window necessary for laboratory light or ventilation.

THE PROBLEM OF EYESTRAIN IN READING OF TESTS

It may be appropriate to comment on the variation in visual acuity among laboratory workers from too rapid or too marked a contrast in laboratory illumination. As with other instruments like the microscope, which require concentration on focusing, eyestrain can result from close observation of tiny particles in flocculation tests. Eyestrain has two frequent causes—lack of exercise of lenticular accommodation, and too marked a contrast between general laboratory illumination and illumination within an instrument such as the microscope.⁵ Similar conditions obtain when reading fine, colorless precipitates of flocculation tests. If retinal adaptation between low laboratory room lighting and too bright instrument illumination becomes too extreme in range, visual fatigue and subsequent impairment of accuracy in reading flocculation tests may follow. Where one individual may have to read, as is not unusual, up to several hundred sets of Kahn tests, the errors arising from flagging visual accommodation may be an important justification for a second reading of the test by the same individual ten minutes later or by a checking associate.

Since this device is designed to minimize glare and to provide critical standard illumination by side-lighting, eyestrain as a procedural error is largely combated.

SUMMARY

A device is described which can be simply constructed. It has two main advantages:

- a. A constant light-source for reading tube flocculation tests.
- b. The convenience of using the Kahn rack without removing the individual tubes.

The factor of eyestrain in reading of serologic flocculation tests is discussed. The Kahn viewing device presented attempts to minimize this source of error.

REFERENCES

1. Todd, J. C., and Sanford, A. H.: *Clinical Diagnosis by Laboratory Methods*, Philadelphia, W. B. Saunders Co., ed. 10.
2. Bray, W. E.: *Synopsis of Clinical Laboratory Methods*, St. Louis, The C. V. Mosby Co., ed. 2.
3. Stitt, E. R., Clough, P. W., and Clough, M. C.: *Practical Bacteriology, Haematology and Animal Parasitology*, Philadelphia, The Blakiston Co., ed. 9.
4. Lowry, E. M.: Screen Brightness and the Visual Functions, *J. Soc. Mot. Pict. Eng.* 26: 491-504, 1936.
5. Hamly, D. H.: Efficient Light Sources in Photomicrography, *Am. Annual of Photography*, Am. Photo. Pub. Co., Boston 57: 58-70, 1943.

THE MACROSCOPIC DETERMINATION OF BLOOD GROUPS A AND B

CAPT. LAWRENCE I. SWAN, M.C., A.U.S.

THE present emergency has necessitated the blood grouping of large numbers of individuals, both civilian and military. It is of paramount importance that the methods used be as rapid as compatible with a high degree of accuracy. In Britain blood group determinations have been performed on a large scale also, and a blood transfusion research committee appointed by the Medical Research Council has published recommended methods now to be followed by all transfusion services of Britain.¹

The method herein described was evolved after many trials and has been found to be highly satisfactory because of its simplicity, rapidity, and accuracy. An effort to determine the accuracy of the typing, resulted in the calculation of the incidence of a large number of blood groups determined by this method.

PREPARATION OF TYPING SERUM

It is well known that the agglutinin titer in individual serum varies markedly, and Thalhimer and Myron² found a range from 1:7 to 1:448. Pooling of serum is obviously the most economical of time and equipment to obtain a typing serum of average iso-agglutinin level.

Each type of serum is obtained from 6 or more healthy, young adult males. Blood from each individual is kept separate in 50 c.c. centrifuge tubes and allowed to coagulate in a refrigerator overnight. The tubes are then reamed and centrifuged, and each tube is retyped before the serum is removed and pooled.

A 5 per cent phenol preservative in physiologic saline is added to the serum in the proportion of 1:9. This results in an immediate precipitation of protein which redissolves, to a large extent, upon the complete addition of the phenol. The protein precipitation may be partially eliminated by agitation of the serum as the phenol is added.

The pooled serum is divided into 3 c.c. amounts in Wassermann tubes, tightly stoppered and stored in a refrigerator. One drop of methylene blue is added to each tube of Type A serum for the purpose of differential coloring. Immediately before use, an equal amount of physiologic saline is added to the serum. Specimens of serum stored in excess of 6 months have not decreased in titer or agglutination time.

TITRATION OF TYPING SERUM

It has been found that the results of various techniques of titration vary considerably. The method used here involves pooled sensitive cells as well as pooled serum. The final measure of a successful typing serum is not the titer

of agglutinins alone, but the rapidity with which macroscopic clumping occurs, for there is little correlation between titer and agglutinating time. At the time blood is drawn approximately a 2.5 per cent suspension of cells in physiologic saline is made from each donor in separate tubes. The content of these tubes is pooled prior to the titration. The cells not incorporated in the clot may also be used after the serum has been removed.

The details of the titration technique are similar to those described by Wiener.³ The final tube is the dilution in which clumps of 3 to 4 cells may be detected microscopically. The titer of Type A averages 1:32 and of Type B, 1:128 by this method, and definite clumping occurs within 15 seconds.

METHOD OF BLOOD GROUPING

Our experience, and that of others,⁴ would indicate that the macroscopic method of blood typing adapts itself satisfactorily to conditions necessitating speed, accuracy and economy of equipment.

A piece of plate glass divided into forty $\frac{3}{4}$ inch squares is placed on a white background. Two or three drops of diluted serum are placed on the plate in the rows labeled A and B respectively. To this is added a small amount of whole blood from the finger which is pricked by an assistant at the left of the individual adding blood to the serum to make the mixture a light pink, thus aiding in the elimination of pseudo-agglutination. The blood types are read and recorded at the end of three minutes and checked after the elapse of seven minutes.

INCIDENCE OF BLOOD GROUPS

A statistical analysis of the blood groups determined by the described method was found to be within the range accepted by most authorities. A total of 65,350 blood groups were studied. Of this number 61,561 were a heterogeneous group of Caucasian males, and 3,789 were Negro males.

The incidence and distribution of blood groups is shown in the accompanying table. The increase in the percentage of Type B at the expense of Type A among those of the Negro race is noteworthy and has been discussed previously by anthropologists.

DISTRIBUTION AND INCIDENCE OF BLOOD GROUPS A AND B

	O		A		B		AB		TOTAL	
		%		%		%		%		%
Negro	1,858	49.03	1,048	27.65	780	20.58	103	2.71	3,789	99.97
White	28,683	46.59	25,042	40.67	5,988	9.72	1,848	3.00	61,561	99.98
Combined	30,541	46.73	26,000	39.92	6,768	10.35	1,951	2.98	65,350	99.98

SUMMARY

1. The preparation of pooled typing serum for the determination of blood groups A and B is described.
2. Phenol in physiologic saline is used as a preservative.
3. The technique of titration involves the use of a pooled suspension of red cells. Type A averages 1:32 and Type B averages 1:128.

4. There is little correlation between agglutinating time and agglutinin titer.

5. The blood groups are determined macroscopically on a plate glass divided into forty $\frac{3}{4}$ inch squares.

6. A total of 65,350 blood groups determined by this method were studied, and the incidence of Caucasian, Negro and the combined types was within acceptable percentages.

REFERENCES

1. Medical Research Council War Memorandum No. 9, The Determination of Blood Groups. Published by His Majesty's Stationery Office, London, 1943.
2. Thalhimer, William, and Myron, S. A.: Globulin Fractions of A and B Agglutinating Serums for Blood Typing, J. A. M. A. 118: 370, 1942.
3. Wiener, A. S.: Blood Groups and Blood Transfusions, Charles C Thomas, 1943, p. 17.
4. Sappington, S. W.: Macroscopic Blood Typing, J. LAB. & CLIN. MED. 28: 1752, 1943.

STABILIZING SERUM FOR BLOOD TYPING

TITUS B. RAY, B.S., AND TRUMAN NELSON, A.B., JACKSON, MICH.

HOSPITAL laboratories, clinics, and private doctors who do a large amount of blood typing, usually have difficulty in preserving blood serum which has been collected and pooled from various sources. The following technique has proved very reliable and satisfactory over a period of the past five years in which it has been employed.

The only special material required for the following procedure is cellophane tubes which may be secured through almost any laboratory supply company for a few cents. There are two types of moisture-proof cellophane tubes which may be used for dehydrating the serum. One, a 29 x 200 mm. tube which is closed at one end; the other, a plain cellophane tubing which may be converted into a U-shaped tube and suspended by clamping both ends to a support. This method of suspension allows free circulation of air during the process of dehydration. Preference is given to dehydration by fanned air for it has been found more satisfactory.

PROCEDURE FOR DEHYDRATING SERUM

1. Collect type two (2) and type three (3) serum over a period of three weeks or more and pool in containers according to the corresponding type.

2. Pour type two (2) serum into one cellophane tube and type three (3) serum into a similar cellophane tube.

3. These tubes are suspended on a support directly in front of an electric fan, approximately two feet away. Leave the tubes in this position until the serum has become completely dehydrated by air from the fan. The dry serum may remain in the cellophane tubes, stored in an ice box until ready for use.

PREPARING SERUM FOR USE

1. Loosen the dehydrated serum from the cellophane tubes by rotating in the palm of hand. Weigh out three (3) grams each of type two (2) and type three (3) dehydrated serum and pour into Pyrex flasks bearing respective labels.

2. Add 35 c.c. of physiologic saline to each flask and rotate five minutes until thoroughly mixed.

3. Place both flasks in H₂O bath at 56° C., rotating occasionally until the serum is completely dissolved. Keep in icebox when not in use. When doing blood typings with dehydrated typing sera, use the same as when employing routine typing sera.

ADVANTAGES

1. Serum prepared this way keeps indefinitely. It has been used up to eight months with accurate results.

2. No preservative is required. Foreign matter does not accumulate as with regular blood typing serum which is pooled from various sources.

3. It is accurate, time saving, very inexpensive, and an advantage to all laboratories, large or small.

DETERMINATION OF PHENOL COEFFICIENTS IN PRESENCE OF SURFACE TENSION DEPRESSANTS

WALTER C. TOBIE, PH.D., AND MARTHA L. ORR, B.S., STAMFORD, CONN.

USING the Food and Drug Administration (F. D. A.) method¹ for determining phenol coefficients, it was found that Aerosol OT (di-2-ethylhexyl sodium sulfosuccinate) had a distinct action in potentiating the germicidal (or bacteriostatic) action of phenol, cresol, and phenylmercuric nitrate against *Staphylococcus aureus* at 37° C. Details will be published later. Early in the work, it was noticed that although the potentiation was very evident, the results of the inoculation from the medication mixture were very erratic, particularly at the time when killing began to be fairly complete. The results checked poorly in repeat determinations, higher dilutions of the potentiated germicides occasionally appearing to kill the bacteria when lower dilutions did not. Since it was noticed that the drops of mixture taken for inoculation were greatly reduced in size if Aerosol OT was present, the effect of the reduction in volume of inoculum was studied.

The standard loop used for inoculating in the F. D. A. method for phenol coefficients consists of a No. 23 Brown and Sharpe gauge platinum wire, with a loop 4 mm. in inside diameter, bent at a slight angle to the straight part of the wire, which is set in a handle or holder. In making inoculations, the tube of medication mixture (containing 5.0 e.e. of diluted germicide and 0.5 e.e. of a 24-hour culture) is held at an angle so that the loop can be lifted perpendicularly from the surface of the liquid at given intervals of time, before being transferred to the culture tube.

Under normal conditions, it is possible to obtain drops of fairly uniform size and volume. This is not the case in the presence of appreciable amounts of Aerosol OT, since the reduced surface tension causes the formation of drops which are not uniform in size, but which on the average are much thinner and flatter than usual, amounting to possibly 10 to 20 per cent of the volume of normal sized drops. Aerosol OT also usually produces a foam over part of the surface of the medication mixture, and if a drop of inoculum is lifted through this foam, its volume is reduced to a thin film like a soap bubble. When such films break (as is often the case), the remaining liquid amounts to only a few minute droplets along the wire of the loop.

Thus with reduced surface tension, it is apparent that the number of bacteria taken for inoculation is reduced in proportion to the diminished size of the drop. When large numbers of viable bacteria are present, the difference in size of drops is of little importance. The main error occurs after the numbers of bacteria have been greatly reduced by the action of the germicide. If, for example, only 5 viable cells remain in the average normal-sized drop, the inocula-

From the Research Laboratories, American Cyanamid Co., Stamford, Conn.
Received for publication, March 27, 1944.

tion will give a distinct growth. But if the size of the drop is reduced to 10 per cent of normal volume, then statistically the average drop of reduced size will contain only half a viable bacterial cell, so that some of the drops will contain no living bacteria at all, and will give no growth. Since the drops are not uniformly reduced in size by the Aerosol OT, the irregular results are readily explained.

Although the action of surface tension depressants in diminishing the volume of inoculum is of considerable importance, it seems to have attracted little or no attention. Thus Gershenfeld and collaborators²⁻⁵ who have done most of the studies on potentiation of germicides by surface tension depressants, used the standard F. D. A. methods in most of their work, but do not mention any reduction in drop size.

To determine the size of the standard drop, 10 drops of distilled water were carefully taken on a standard loop, and struck off into a homeopathic vial. The increase in weight of the vial was then determined. The total weight of the first 10 loopfuls was 0.1914 Gm., that of the second 10 loopfuls was 0.2036 Gm. Thus the weight of the average drop is 0.0197 Gm. or very close to 0.02 Gm. (0.02 c.c.).

To obtain 0.02 c.c. of medication mixture for inoculation in the presence of Aerosol OT, sterile 0.2 c.c. Kahn pipettes, graduated to 0.001 c.c., were used instead of the loop in all inoculations. With such pipettes, 0.02 c.c. of medication mixture was easily taken, whether or not Aerosol OT was present.

Using the pipette technique, Aerosol OT did not appear to potentiate the germicides to quite as great a degree as when the loop was used, but different runs gave better checks, so that the results were much more consistent. Thus it was possible to measure the true potentiation, rather than the potentiating action plus the effect of a considerable but variable reduction in the volume of the inoculum.

SUMMARY

In the presence of substances such as Aerosol OT, which materially reduce surface tension, the size of the drop taken for inoculation on the loop in the standard F. D. A. phenol coefficient method is greatly, but not uniformly, reduced. Consistent results are hard to obtain, and apparent potentiation is greater than is actually the case. These difficulties may be overcome by taking 0.02 c.c. of the medication mixture in a sterile pipette for inoculations.

REFERENCES

1. Ruehle, G. L. A., and Brewer, C. M.: U. S. Food and Drug Administration Methods of Testing Antiseptics and Disinfectants. U. S. Department of Agriculture, Circular No. 198, 1931.
2. Gershenfeld, L., and Perlstein, D.: Significance of the Hydrogen-Ion Concentration in the Evaluation of the Bactericidal Efficiency of Surface Tension Depressants, A Preliminary Study on Aerosol O.T., *Am. J. Pharm.* 113: 88, 1941.
3. Gershenfeld, L., and Witlin, B.: Surface Tension Reducents in Bactericidal Solutions: their *in Vitro* and *in Vivo* Efficiencies, *Am. J. Pharm.* 113: 215, 1941.
4. Gershenfeld, L., and Perlstein, D.: The Effect of Aerosol OT and Hydrogen-Ion Concentration on the Bactericidal Efficiency of Antiseptics, *Am. J. Pharm.* 113: 237, 1941.
5. Gershenfeld, L., and Milanick, V. E.: Bactericidal and Bacteriostatic Properties of Surface Tension Depressants, *Am. J. Pharm.* 113: 306, 1941.

CHEMICAL

A METHOD FOR THE RECOVERY OF PENICILLIN FROM THE URINE

LAWRENCE H. SOPHIAN, M.D., SENIOR SURGEON (R) USPHS
U. S. MARINE HOSPITAL, STATEN ISLAND, N. Y.

IN THE course of introducing penicillin into clinical use in this hospital, it became apparent that the supply would be limited for a considerable time. Since it is known from previous reports (1) that over 50 per cent of a dose of penicillin administered intravenously appears in the urine during the first hour thereafter, we decided to attempt extraction and reclamation of excreted penicillin and to test its efficacy as a therapeutic agent. The principles of extraction are identical with those followed in the manufacture of penicillin. The problem is somewhat easier than that of extraction from cultures of penicillium because the urine contains none of the toxic and pyrogenic fractions which are a source of considerable difficulty. Urinary content of penicillin varies with the size of the dose. When 10,000 units are being administered at intervals of three hours by the intramuscular route, the urine contains from 25 to 40 units per cubic centimeter. With larger dosage, we have found some samples of urine containing 125 units per cubic centimeter. Such concentrations enable one to make a satisfactory extraction of the major portion of penicillin by a single acidification and elution with an organic solvent immiscible with water. For the sake of simplicity, we have confined ourselves to such a procedure, although a small increase in yield results from multiple extractions.

A large container is kept in the icebox for collection of urine from patients under treatment with penicillin. The collections may be made over a period of forty-eight hours without loss of penicillin.

The urine is acidified with concentrated hydrochloric acid and brought to a pH of 2 as determined by the color change produced with methyl violet indicator. Approximately six to seven cubic centimeters of acid are needed for each liter of urine.

Amyl acetate is added to the urine in a proportion of one part to three of urine. A reservoir bottle with an outlet at the bottom serves as a convenient container for mixing. The mixing of the urine and acetate is accomplished by inversion of the bottle twelve or fifteen times. Too much agitation should be avoided to prevent foaming.

The bottle is placed in the icebox for four hours to allow separation of the urine and acetate. The urine is then drawn off through the lower outlet, leaving the penicillin dissolved in the amyl acetate. Any foamy middle layer is passed through several thicknesses of gauze and from it as much amyl acetate recovered as possible.

The amyl acetate extract is now transferred to a separatory funnel of one liter capacity. Approximately 20 c.c. of phosphate buffer solution are added to it for each liter of amyl acetate. The buffer is prepared by mixing approximately one part of sodium acid phosphate to two parts of *disodium* phosphate by weight, adjusting to pH 7. After mixing the buffer solution with the amyl acetate, some color (representing the pigment which occurs along with penicillin) will be seen to transfer to the buffer solution. The pH of the buffer solution is now adjusted to pH 6.5 by gradual additions of 5 per cent sodium carbonate solution or of two normal sodium hydroxide solutions. By using the latter, less dilution will occur so that a more concentrated final product will result, but more care must be exercised in order not to pass into the alkaline range. The pH is determined by testing of single drops of the solution with bromthymol blue indicator, agitating thoroughly after each addition. When pH 6.5 is reached, practically all the color will have passed from the amyl acetate solution into the buffered aqueous solution. The transfer of penicillin is almost quantitative. The separatory funnel is placed in the icebox for six hours to complete the separation and the amyl acetate is then drawn off. The amyl acetate may be saved and used again.

Additional fractions of amyl acetate extract may be added to the aqueous solution of penicillin in the funnel so that greater concentration may be effected into the same small volume; each time the minimal volume of alkaline solution is added to return the reaction to pH 6.5. With increasing concentration of the extract, the color of the solution approaches that of coffee.

Testing for activity of penicillin is done by the dilution method against staphylococcus H, as described by Hamre, Rake, et al.² A satisfactory product is then Seitz filtered into sterile vials. Useful concentrations of three thousand units of penicillin or more per cubic centimeter may be obtained. The yield with this method has been approximately thirty per cent of the amount administered to the patients. For every one million units given the patient, three hundred thousand units can be recovered in a form suitable for re-use.

A test for toxicity is made by injecting one hundred thousand units intravenously into one or more rabbits. Thus far, no toxicity has been encountered. Since the titration of the extract for activity and the testing for toxicity represent the most involved steps in the process, it is suggested that a number of extractions be pooled and concentrated as much as possible and that a sample from the total be titrated and tested. Two hundred cubic centimeters or more of the concentrated deeply colored extract are usually collected by us before testing.

The aqueous solution of the filtered extract is kept in an icebox in vials until used. The extract has been used in doses equivalent in amount to the commercial product in patients with gonorrheal urethritis in this hospital with equal effectiveness and without reaction. Storage for sixty days at -10° C. demonstrated complete maintenance of anti-biotic activity.

Extraction of penicillin from urine and its re-use in patients was reported by Abraham, E. P., Chain, E., et al.,³ and they noted that pyrogens contained in the original penicillin preparation were removed by passage through the body. Their paper contains no description of the technique of recovery.

SUMMARY

In order to extend the available supply of penicillin, adaptations have been made of the methods employed in penicillin production and a simplified technique is described by which penicillin can be reclaimed from the urine of patients receiving it. Extraction and titration by this method can be performed with apparatus and materials available in an ordinary hospital laboratory. An average yield of 30 per cent of the administered amount has been obtained, and the antibiotic and pharmacologic effects have been identical with those of commercial penicillin.

REFERENCES

1. Rammelkamp, C. H., and Bradley, S. E.: Excretion of Penicillin in Man, *Proc. Soc. Exper. Biol. & Med.* 53: 30, 1943.
2. Hamre, D. M., Rake, G., McKee, C. M., and MacPhellamy, H. B.: The Toxicity of Penicillin, *Am. J. M. Sc.* 206: 642, 1943.
3. Abraham, E. P., Chain, E., Fletcher, C. M., Gardner, A. D., Heatley, N. G., Jennings, M. A., and Florey, H. W.: Further Observations on Penicillin, *Lancet* 2: 177, 1941.

THE DETERMINATION OF TOCOPHEROL IN BLOOD SERUM

ANN S. MINOT, PH.D., NASHVILLE, TENN.

MORE determinations of the tocopherol content of human blood serum are needed in studies of vitamin E in human nutrition and in attempts to demonstrate whether or not lack of vitamin E may play a role in certain pathologic conditions. The only figures yet available were contributed by Wechsler, Mayer and Sabotka,^{1, 2} who reported comparative studies of the concentrations of tocopherol in the blood sera of normal persons and of patients with amyotrophic lateral sclerosis and with various myopathies. In a recent attempt to make direct application of the procedure described by Mayer and Sabotka,³ we encountered so many difficulties that it seems worth while to record here the modifications we have found necessary in order to obtain satisfactorily quantitative results.

The method best suited for the determination of small amounts of tocopherol appears to be the photoelectric measurement of the color produced when tocopherol reacts with ferric iron in the presence of α - α -dipyridyl in an organic solvent. This sensitive color reaction was originally described by Emmerie and Engle⁴ and the same colorimetric principle was used by Mayer and Sabotka³ in their study of blood serum and by Devlin and Mattill⁵ and Hines and Mattill⁶ for measuring the amount of tocopherol in tissues. The reaction has the advantage of great sensitivity and, at the same time, the disadvantage of lack of specificity for tocopherol. The variety of modifications introduced since the method was originally described represents attempts (a) to work out optimum conditions so that full advantage may be taken of the delicate color reaction, and (b) to isolate quantitatively the tocopherol present in biologic material from other substances which would contribute to or interfere with the amount of color produced in the final colorimetric measurement.

COLORIMETRIC DETERMINATION OF PURE ALPHA TOCOPHEROL

The color reaction, as originally employed by Emmerie and Engle⁴ and later used by Mayer and Sabotka,³ was carried out in a mixture of alcohol and benzene. Although quantitative results were obtained under these conditions when certain precautions were observed, the use of alcohol as a solvent has distinct disadvantages. When ferric chloride and α - α -dipyridyl are brought together in absolute alcohol, a deep coloration gradually develops, especially on exposure to light. Thus, the two solutions have to be kept separate until used, but even with this precaution there is some development of color when benzene and alcoholic solutions of ferric chloride and dipyridyl are brought together in a blank determination. The substitution of glacial acetic acid for

From the Department of Pediatrics, Vanderbilt University School of Medicine.
This work was aided by a grant from the John and Mary R. Markle Foundation.
Received for publication, May 8, 1944.

absolute alcohol by Devlin and Mattill⁵ acting on a suggestion by Merek and Company overcomes this difficulty. A stable reagent can be made by dissolving ferric chloride and α - α -dipyridyl in glacial acetic acid. The solution has only the yellow color imparted by the ferric chloride and there is no change in color on long standing or exposure to light. Devlin and Mattill⁶ carried out the colorimetric step by the addition of this reagent to solutions of tocopherol in Skellysolve B. The reagent is also readily miscible with benzene and no color is produced in blank determinations. Since benzene was more readily available to us it was adopted in preference to Skellysolve B as a solvent for tocopherol in all our analyses. When the reagent is added to a solution of alpha tocopherol in benzene a pinkish orange color develops which reaches maximum intensity in about ten minutes. A Cenco-Sheard Spectrophotometer was used and readings were made at a wave length of 520 m μ after allowing ten minutes for color development.

Under these conditions the Beer-Lambert law obtained over a range of from 20 to 250 gamma of pure alpha tocopherol in a final reaction volume of 15 c.c. (representing 5 c.c. of a benzene solution of the tocopherol and 10 c.c. of the glacial acetic acid reagent). By plotting the logarithmic values of the transmission against concentration of tocopherol, a straight line of reference was readily obtained or, alternatively, an appropriate function of the extinction coefficient for the ferrous iron-dipyridyl complex could be derived for the calculation of tocopherol in unknown solutions. Occasional rechecking of these values with fresh solutions of alpha tocopherol at intervals of a few weeks has given practically identical figures. This constancy of results has served as reassuring evidence of the reliability of the photometric analysis in the absence of interfering substances.

THE QUANTITATIVE ISOLATION OF TOCOPHEROL FROM BLOOD SERUM

Preliminary to the colorimetric determination the tocopherol must be extracted from blood serum without loss and separated quantitatively from other substances which would interfere with the final color reaction.

Emmerie and Engle, and later, Mayer and Sabotka, used ether for the extraction of tocopherol from blood serum previously treated with formaldehyde, ethyl alcohol, and dilute alkali. Devlin and Mattill used a mixture of Skellysolve and alcohol for tissues treated in a Waring Blender. In this way they avoided saponification with alkali, which involves the possibility of loss in view of the recognized instability of tocopherol in alkaline solutions. Actually, however, in working with blood serum, we could demonstrate no loss of tocopherol in the brief exposure to dilute alkali involved in the extraction process of Emmerie and Engle and have continued to use this method.

Regardless of which method of extraction is employed, carotenoids and cholesterol are simultaneously extracted and must be removed before the tocopherol can be accurately determined colorimetrically. Selective adsorption of vitamin A and other carotenoids on Floridin X S earth has, in the hands of some investigators, proved a successful means of avoiding the interference from these constituents. Others have found this procedure unsatisfactory, due

possibly to slight differences in the adsorptive properties of different preparations of Floridin earth and to the fact that the fundamental principles governing the success or failure of this selective adsorption have not been accurately defined. Our own experience has been that, despite repeated attempts with several samples of Floridin earth* and careful attention to the details of the procedures described by those who have used it successfully, we have never been able to remove carotenoids without a varying, but always significant, loss of tocopherol. Like Devlin and Mattill, we have found the chemical removal of these contaminants by treatment with 85 per cent sulfuric acid, as described by Parker and McFarlane,⁸ a consistently reliable procedure. This treatment does not obviate the interference due to cholesterol, but after the carotenoids have been removed, Devlin and Mattill have shown that cholesterol can be removed without loss of tocopherol by adsorption from benzene on Florisil (Supersorb). After purification by the acid treatment and subsequent passage through adsorbent columns of Florisil, a solution of tocopherol is obtained essentially free from interfering contaminants.

Whatever procedures are adopted, precautions have to be taken throughout the various manipulations to avoid oxidative loss of tocopherol. All evaporations should be carried out *in vacuo*, and flasks, adsorption columns, etc., should be flushed out with nitrogen before the introduction of tocopherol solutions. Direct sunlight should be avoided but the more elaborate precautions of the use of amber glass and a dark room as described by Mayer and Sabotka have not been found to increase the accuracy of the modified procedure which we have adopted.

DETAILS OF METHOD FINALLY USED FOR STUDIES OF BLOOD SERUM

A detailed account of the procedures we have used for the determination of tocopherol in serum follows. Summarized in barest outline, the method combines the extraction process of Emmerie and Engle as modified by Mayer and Sabotka with the Parker and McFarlane method for the removal of vitamin A and other carotenoids, and the adsorptive removal of cholesterol by means of Florisil as described by Devlin and Mattill. The final photometry is carried out on a benzene solution of tocopherol with a solution of ferric chloride and α - α -dipyridyl in glacial acetic acid.

The following reagents are required:

Peroxide-free ether, redistilled from sodium.

Benzene C.P. reagent.

Petroleum ether, purified as directed by Parker and McFarlane.⁸

95 per cent ethyl alcohol.

Formaldehyde, commercial 37 per cent aqueous solution neutralized with NaOH.

0.2 N KOH in distilled water.

*Kindly furnished for experimental trial by The Floridin Company, Warren, Pennsylvania.

2 per cent KOH in distilled water.

1 per cent (by volume) H_2SO_4 in water containing 1 per cent cadmium sulfate.

0.5 per cent Na_2SO_4 in distilled water.

85 per cent H_2SO_4 , 85 c.c. of concentrated H_2SO_4 , specific gravity 1.98 diluted to 100 c.c. with distilled water.

Iron-dipyridyl color reagent, 125 mg. of ferrie chloride and 250 mg. of α - α -dipyridyl are dissolved in 500 c.c. of C.P. glacial acetic acid.

Florisol,* purified according to the directions given for Floridin by Emmerie and Engle.⁹

Nitrogen, commercial tank.

PROCEDURE

To a measured 5 to 10 c.c. volume of serum (made to 10 c.c. by the addition of water), in a 250 c.c. glass-stoppered separatory funnel, are added 5 c.c. of 0.2 N KOH, 15 c.c. of formaldehyde solution and 15 c.c. of 95 per cent alcohol, and the mixture thoroughly shaken. Extraction is then carried out with three successive 50 c.c. portions of peroxide-free ether. After each extraction, 10 c.c. of 95 per cent alcohol are added to the aqueous solution preliminary to the next addition of ether. After the third extraction, the aqueous layer is discarded. The combined ether extracts are then washed in a separatory funnel, once with 25 c.c. of 2 per cent KOH, twice with 15 c.c. portions of 1 per cent H_2SO_4 containing cadmium sulfate, and three times with 25 c.c. portions of 0.5 per cent Na_2SO_4 . The shaking should be gentle, especially in the final washings, or troublesome emulsions may form. The washed ether is dried for an hour or more over anhydrous sodium sulfate. At this stage the ether extract is perfectly clear and usually has a yellow tint due to the presence of carotenoids.

After drying, the ether solution is quantitatively transferred to a suction flask by filtering through a layer of anhydrous sodium sulfate. Several portions of pure dry ether are used for rinsing the original flask and filter; and these washings are combined with the filtrate. The suction flask is then supported in a warm bath and the ether evaporated *in vacuo*. When only a small volume of ether remains, repeated small additions of benzene are made as the evaporation continues in order to drive off the last traces of water and alcohol. This is conveniently accomplished by using a suction flask fitted with a small separatory funnel for the repeated addition of benzene. (In arranging apparatus for this or any other step in the method, the use of rubber stoppers or connections must be avoided as organic solvents remove material from rubber which later interferes with the accuracy of the method). Usually about 30 to 40 c.c. of benzene are required and this is added in portions of 5 to 10 c.c. until no turbidity results from the addition of benzene to a small residue of fluid in the flask. Evaporation is then continued to dryness. At this point there is a small, yellowish residue left in the flask. The reduced pressure is maintained while the flask

*Purchased from the Floridin Company, Warren, Pa.

is thoroughly cooled, and then an accurately measured 15 c.c. volume of petroleum ether is added which quickly and completely dissolves the residue, and the solution is promptly poured into a glass-stoppered tube which can be centrifuged. Three cubic centimeters of 85 per cent sulfuric acid are added and the tube stoppered and inverted several times until the now brown aqueous layer tends to settle quickly to the bottom of the tube. The stoppered tube is then centrifuged to complete the separation. The clear colorless petroleum ether layer is then transferred as completely as possible to a second glass-stoppered centrifuge tube and washed by inverting several times after the addition of 5 c.c. of 2 per cent KOH. After centrifuging, as large an aliquot as possible of the petroleum ether solution is measured into a small flask. Usually 10 c.c., representing two-thirds of the original 15 c.c. of the petroleum ether solution which contained the entire residue from the sample of serum, can be obtained without difficulty. Obviously all transfers and manipulations of the petroleum ether solution should be carried out as expeditiously as possible and in cool surroundings in order to avoid evaporation of the solvent, and hence change in concentration of dissolved tocopherol, before the aliquot is measured for further treatment.

At this stage the petroleum ether contains tocopherol which is free from vitamin A and carotenoids but is still contaminated with significant amounts of cholesterol. Since the adsorption of cholesterol on Florisil is only satisfactory from a solution in benzene, the aliquot of petroleum ether is evaporated to dryness *in vacuo* and the slight residue is taken up in 5 c.c. of benzene. In the meantime, adsorption columns of Florisil are prepared. Previously purified dry Florisil is placed in glass tubes to form a column approximately 80 by 12 mm. Before use, the Florisil is washed once or twice with pure benzene, and nitrogen gas is run through the column to displace air. The 5 c.c. of benzene solution is then run through the column and is followed by about 25 c.c. of benzene added in portions of 5 c.c. each to the original flask and thence through the column. The combined solution and washings are collected in a small suction flask and evaporated to a volume of about 5 c.c. This is then washed through a second adsorption column of Florisil in exactly the manner just described. Probably for most bloods one passage through Florisil is adequate, but occasionally in dealing with a serum of high cholesterol content a determination was ruined by small amounts of cholesterol which persisted after a single treatment; therefore, the uniform procedure of using two columns was adopted.

When the combined benzene washings from the second adsorption column are evaporated to dryness, the residue is practically invisible. After cooling, this is taken up in 5 c.c. of benzene, and 10 c.c. of the glacial acetic acid reagent are added and the color allowed to develop for ten minutes before the photometric reading is made. A blank solution is prepared simultaneously by the addition of 10 c.c. of the reagent to 5 c.c. of pure benzene. By reference to a calibration curve previously obtained by analysis of pure alpha tocopherol solutions, and appropriate correction for the original volume of serum and the size of aliquot used, the tocopherol content of 100 c.c. of serum can be readily calculated from the observed transmission. As pointed out by Hines and Mattill, the practice of taking a second reading twenty to thirty minutes after the color reagent is

added affords a convenient means of checking the efficacy of the steps for removal of interfering substances. Pure tocopherol solutions show maximum color development in ten minutes, and there is no appreciable change during the next ten to twenty minutes. When impurities are contributing to the color, different time relationships are encountered and there is significant decrease in transmission noted in the later readings as compared to the ten-minute reading. When such differences are noted, the results are unreliable and should not be accepted as true tocopherol values.

Needless to say, before studies of serum are undertaken, blank determinations should be run in which 10 c.c. of water are substituted in place of serum and carried through the entire procedure. Entirely satisfactory blanks should neither cause any development of color with the iron-dipyridyl reagent nor interfere with the development of the full color value when a known amount of tocopherol is measured in the presence of the residue from a blank determination. Actually, a slight amount of color, equivalent to 1 to 3 gamma of tocopherol usually develops in a blank determination in spite of carefully purified reagents. Blanks should be run whenever new lots of reagents are introduced, and a correction made for any slight introduction of extraneous color.

In Table I are collected data which demonstrate the satisfactory recovery of pure alpha tocopherol carried through various steps of the procedure in the manner just described. The figures in the last group also indicate the efficiency of the Parker-McFarlane treatment followed by double adsorption of Florisil in avoiding the interference due to carotenoids and cholesterol.

TABLE I
RECOVERY OF PURE TOCOPHEROL CARRIED THROUGH VARIOUS PROCEDURES

TEST PROCEDURE	TOCOPHEROL RECOVERED (GAMMA)	TOCOPHEROL PRESENT (GAMMA)	PER CENT RECOVERED
α tocopherol in benzene carried through two Florisil columns	(a) 95	100	95
	(b) 102	100	102
α tocopherol carried through acid treatment and two Florisil columns	(a) 100	100	100
	(b) 97	100	97
Mixture of α tocopherol, carotene, and cholesterol, carried through acid treatment and two Florisil columns	(a) 108	100	108
	(b) 97	100	97
	(c) 102	100	102
	(d) 47	50	94

Table II contains the results of preliminary studies with blood serum. Essentially the same values in terms of milligrams of tocopherol per 100 c.c. of a given serum were found whether the determination was run on a 5 or 10 c.c. sample. In spite of the readily reproducible results there should be some reservation in the complete acceptance of the values obtained as the true level of alpha tocopherol. Other tocopherols with less biologic activity than alpha tocopherol give the same color reaction and would not have been removed in the procedures incorporated in the method. Furthermore, although several known interfering substances have been successfully eliminated, there has been no direct proof that others not yet recognized may still be involved. Fairly good evidence that the principal color-producing factor measured is alpha tocopherol is

afforded by the very low values obtained in the serum of rabbits that are seriously deficient in vitamin E, and the prompt increases which are always noted when liberal amounts of vitamin E are restored to such animals. Satisfactory recoveries could be made of alpha tocopherol added to blood serum.

TABLE II
PRELIMINARY BLOOD STUDIES AND RECOVERIES OF ADDED TOCOPHEROL

MATERIAL STUDIED	VOLUME SERUM USED FOR DETER- MINATION	TOCOPHEROL FOUND (MG./100 C.C.)	TOCOPHEROL PRESENT (MG./100 C.C.)	RECOVERY OF ADDED TOCOPHEROL (PER CENT)
Human serum A	10	0.97	-	-
Human serum A	5	1.02	-	-
Human Serum A + 1 mg./100 c.c. of added tocopherol	5	2.09	2.00*	109
Human Serum B	10	1.34	-	-
Human Serum B	8	1.32	-	-
Human Serum B + 1 mg./100 c.c. of added tocopherol	9	2.37	2.33*	104
Human Serum B + 0.5 mg./100 c.c. added tocopherol	5	1.78	1.83*	96
Rabbit Serum No. 1. Dying from E deficiency	10	<0.05	-	-
Rabbit Serum No. 2. E deficient diet. Beginning to show weakness	10	0.43	-	-
Rabbit Serum No. 3. Same rabbit as No. 2 after administration of E	8	1.74	-	-
Rabbit Serum No. 4. E deficient for weeks. Marked muscular weakness	8	0.26	-	-
Rabbit Serum No. 5. Same rabbit as No. 4 after heavy E medication	5	2.76	-	-

*Figure based on average figure without additions, plus added tocopherol.

TABLE III
SERUM TOCOPHEROL LEVELS DETERMINED BY THE PROCEDURE DESCRIBED

SUBJECT	AGE (YR.)	SEX	DIAGNOSIS	SERUM TOCOPHEROL (MG./100C.C.)
H. B.	15	M	No disease	0.72
L. J.	11	M	Behavior problem	0.94
J. N.	13	M	Allergy	1.12
M. E.	9	F	No disease	0.88
J. L.	11	M	No disease	0.98
A. G.	5	M	Respiratory infection	1.02
J. C.	5	F	Celiac disease	0.64
F. C.	14	F	Otitis media	0.82
H. B.	9	M	Bronchitis	0.79
D. G.	14	M	Nephritis	1.20
H. J.	21	M	Muscular dystrophy	0.97
H. H.	13	M	Muscular dystrophy	1.02
W. N.	9	M	Muscular dystrophy	1.28
J. A.	6	M	Muscular dystrophy	1.22
J. L.	9	M	Muscular dystrophy	0.73
H. Je.	12	M	Muscular dystrophy	0.80
J. N.	12	M	Muscular dystrophy	0.81

In Table III are presented the results of tocopherol determinations run on the sera of both ill and apparently normal individuals most of whom were in the pediatric age groups. The entire range of values is fairly small and the usual concentration appears to be about 1 mg. of tocopherol per 100 c.c. of serum. In the small group studied there appeared to be no significant difference in the

levels found in sick and well children. Our figures on patients with muscular dystrophy were done in connection with other studies of that disease and their significance has been discussed elsewhere.¹⁰ It is interesting to note the relatively low concentration found in the serum of one child with celiac disease since this patient was found also to have poor absorption of vitamin A. Presumably, conditions unfavorable for the absorption of another fat soluble vitamin would also hinder the absorption of vitamin E.

DISCUSSION

The values for serum tocopherol which we have presented fall within about the same range as those found by Wechsler, Mayer, and Sabotka in adult serum. The fact that two laboratories using different techniques for several steps in the determination have obtained essentially the same results lends considerable support to the probable accuracy of both sets of figures. On this basis there is perhaps little choice between the two methods. In our hands at least, the use of acid for the removal of vitamin A and carotenoids has proved a far less treacherous procedure than selective adsorption on Floridin. Mayer and Sabotka make no mention of the possibility of interference due to cholesterol. Possibly under the conditions of their procedure, cholesterol was removed or had less influence on the final color reaction. Certainly in the procedure which we have used, the removal of cholesterol with the help of Florisil is a vitally important step. At least from the point of view of convenience, the stable glacial acetic acid solution of ferric chloride and α - α -dipyridyl has great advantages over an alcoholic solution of these reagents.

SUMMARY AND CONCLUSIONS

Modifications of several previously described procedures have been combined in a method which employs the iron-dipyridyl reaction for the colorimetric determination of tocopherol in blood serum.

Data are presented which show (a) alpha tocopherol can be carried through the suggested procedures without significant loss, and (b) that tocopherol can be satisfactorily isolated from substances present in the blood serum which would otherwise interfere with the final colorimetric reaction.

As measured by the method described, the tocopherol content of children's blood serum was found to fall within a small range of values approximating 1 mg. per 100 c.c. of serum.

REFERENCES

1. Wechsler, I. S., Mayer, G. G., and Sabotka, H.: Tocopherol Level in Serum of Normals and Patients With Amyotrophic Lateral Sclerosis, *Proc. Soc. Exper. Biol. & Med.* 47: 152, 1941.
2. Wechsler, I. S., Mayer, G. G., and Sabotka, H.: The Tocopherol Level in Human Serum During Oral Tocopherol Therapy, *Proc. Soc. Exper. Biol. & Med.* 53: 170, 1943.
3. Mayer, G. G., and Sabotka, H.: Photoelectric Determination of d-1- α -Tocopherol in Serum, *J. Biol. Chem.* 143: 695, 1942.
4. Emmerie, A., and Engle, Chr.: Colorimetric Determination of α -Tocopherol (Vitamin E), *Rec. Trav. Chim.* 57: 1351, 1938.
5. Devlin, H. B., and Mattill, H. A.: The Chemical Determination of Tocopherols in Muscle Tissue, *J. Biol. Chem.* 146: 123, 1942.

6. Hines, L. R., and Mattill, H. A.: The Chemical Determination of Tocopherols in Liver and Muscle; Tocopherols in Urine and Feces, *J. Biol. Chem.* **149**: 549, 1943.
7. Emmerie, A., and Engle, Chr.: Colorimetric Determination of Tocopherol (Vitamin E). III. Estimation of Tocopherol in Blood Serum, *Rec. Trav. Chim.* **58**: 895, 1939.
8. Parker, W. E., and McFarlane, W. D.: A Proposed Modification of Emmerie's Iron-Dipyridyl Method for Determining the Tocopherol Content of Oils, *Canad. J. Research* **18**: 405, 1940.
9. Emmerie, A., and Engle, Chr.: Colorimetric Determination of Tocopherol (Vitamin E). II. Adsorption Experiments, *Rec. Trav. Chim.* **58**: 283, 1939.
10. Minot, A. S., and Frank, H. E.: Serum Tocopherol: Its Relation to Failure of Vitamin E Therapy for Pseudohypertrophic Muscular Dystrophy, *Am. Jour. Dis. Child.* In Press.

EDITORIAL

Editorial Policy

UNDER the guidance of Dr Victor C. Vaughan, Dr. Warren T. Vaughan, and the Associate Editors who worked so devotedly with them, THE JOURNAL OF LABORATORY AND CLINICAL MEDICINE has served for the past twenty-nine years as a forum for the presentation of clinical investigation and of laboratory techniques. The reasons for beginning the JOURNAL and description of its editorial policy were eloquently stated by the elder Dr. Vaughan in his first editorial: "At present there is a wide chasm between the research man and the practitioner. The work of one supplements that of the other. The world is not benefited nor medicine advanced until the work of both has been done. The basic aim of this JOURNAL will be to bring discovery and its application closer together, to supply the research man with a strictly scientific organ through which he can present the results of his labors, and to suggest to the practitioner how he may use the latest discovery."

Strict adherence to this editorial policy has at times been difficult. With the rapid growth of the medical sciences it often seemed wise to publish manuscripts which were fundamentally biochemical, physiologic, pharmacologic, or bacteriologic in their interest. Their relationship to clinical medicine was not always immediately apparent, although in many instances these investigations later served as the background for new approaches to clinical problems. On the other hand, studies which presented purely clinical observations or case reports often seemed important enough to print even though they described no new principle nor concerned themselves with the pathogenesis or treatment of disease. At first, the section on "Methods" was confined to a discussion of procedures which were of interest to the clinical pathologist or to the practitioner in his own routine laboratory. This policy had to be broadened, however, so that investigators who used either human or animal subjects could be given an opportunity to exchange their experiences with methods of a more purely experimental nature.

There has, therefore, been a gradual broadening of the editorial policy so that papers with a very wide scope of interest were accepted for publication. It is perhaps now necessary to define the boundaries more precisely. The ideal of trying to bring discovery and its application closer together must certainly be preserved. It would seem that this end might best be served: (1) by confining acceptance of experimental papers to those which can properly be included in the broad field of clinical investigation; and (2) by selecting those clinical papers which demonstrate most clearly the application of recent experimental discoveries to the solution of clinical problems. The policy stated above which governs the publication of methods, techniques, and their analyses will be maintained.

C. V. M.

BOOK REVIEWS AND NOTICES

Colorimetric Determination of Traces of Metals. By E. B. Sandell, Ph.D., Assistant Professor of Analytical Chemistry, University of Minnesota, Minneapolis, Minnesota, Interscience Publishers, Inc., 215 Fourth Avenue, New York 3, New York, 1944. Price \$7.00. Cloth with 487 pages.

This book should prove to be a valuable reference work for biochemists and investigators who find it necessary to determine traces of metals in biological materials. It opens with a general discussion of the methods of trace analysis, the problem of separating and isolating traces of substances, and the chemical behavior of the more common colorimetric reagents. The second half of the book is devoted to a presentation of specific methods for individual metals. It is not encyclopedic in its listing of techniques. An attempt is made rather to describe and evaluate a limited number of methods which seem most satisfactory to the author. The treatment of material is so designed as to be of value to industrial as well as to biological chemists. In some instances, consequently, the applications of methods to biological materials are not given in as much detail as a clinical investigator or clinical pathologist might wish. The principles, however, are all clearly stated, and tabulation is made of those substances which interfere with the accuracy of procedures.

The author occasionally makes statements such as the following which make one question his familiarity with the difficulties of doing determinations on biological materials: "The methods now available (for copper) are in the main satisfactory and leave little to be desired." Anyone who has tried to study the copper content of blood realizes how far from the truth this opinion is. However, the reviewer knows of no other work which assembles so compactly and so well the most acceptable methods of colorimetric trace analysis.

C. V. M.

Vascular Responses in the Extremities of Man in Health and Disease. By David I. Abramson, M.D., F.A.C.P. Associate in Charge of Cardiovascular Research, May Institute for Medical Research of the Jewish Hospital, Cincinnati. (Now is a Captain in the Army Medical Corps.) University of Chicago Press, Chicago, Ill. Price \$5.00. Cloth with 412 pages.

The book attempts to assemble and correlate information on peripheral vascular disease and vascular responses which has been widely scattered through the literature. The author devotes a large amount of space to the physiological responses of the peripheral vascular tree. Diseases of the venous and arterial systems are evaluated largely from the physiological rather than from the clinical standpoint. Experiments on vascular responses in the limbs of man are extensively considered; animal experiments are described to fill in where data on man are wanting.

The book opens with chapters on the anatomy and physiology of blood vessels. The responses of the peripheral blood vessels to various pharmacological agents, and in systemic diseases are taken up. There follow considerations of "functional" and organic disease. A final section concerns the methods of treatment of vascular disorders.

This volume is well documented, and is readable. References are included at the end of each chapter, and constitute an extensive compilation of the literature. The index is adequate. In the reviewer's opinion, this work should prove of exceptional value to students and clinicians as a brief, authoritative account of vascular problems.

J. R. S.

Stop Worrying and Get Well. By Edward Podolsky, M.D. Bernard Ackerman, Inc., 381 Fourth Avenue, New York 16, New York. Price \$2.00. Cloth with 124 pages.

CORRESPONDENCE

To the Editor.—In our recent paper entitled "The Apparent Advantage of Frequently Administered Quinine in Avian Malaria Infections" in the JOURNAL, January, 1944, page 43, we made the statement that we were unaware that frequent, to say nothing of continuous, therapy with quinine had ever been given a trial in acute malaria infections in the human being, and that we, therefore, sought to investigate the possible advantages of such a method of treatment by putting the matter to experimental trial in avian infections in the laboratory. Our attempt to effect continuous administration of solutions to canaries having met with failure, we were obliged to resort to administration of the quinine by mouth at two-hour intervals around the clock for the four days during which the therapeutic trials lasted. We have now received a very kind letter from Col. Charles F. Craig, the eminent malariologist and editor of the *American Journal of Tropical Medicine*, who states his feeling that in our search of the clinical literature, we overlooked the fact that frequent quinine dosage was employed in man many years ago and that the matter is specifically discussed in the publications of Marchiafava and Bignami and of himself. In our copy of Marchiafava and Bignami, that was reprinted from *Twentieth Century Practice* for the use of the U. S. Army in 1900, we have failed to find very specific evidence that the Italian authors did use quinine at frequent intervals in the attempt to accomplish a somewhat "continuous" type of therapy, but there can be no doubt that Col. Craig did report very favorably upon a frequent-administration method of treatment in his work *The Malarial Fevers, etc.*, published in 1909. Among his several references to this type of treatment, there appears (page 373) the following unequivocal statement ". . . it is necessary to give quinine in divided doses of from 0.3 to 0.6 Gm. (5 to 10 grains) at intervals of four to six hours until the temperature reaches normal, and to increase the number of doses if experience shows that

the quantity advised is insufficient." It was our misfortune not to have seen this statement in Col. Craig's valuable book before our paper appeared, and we are therefore writing this letter to place on record our recognition of the fact that Col. Craig did indeed advocate the frequent administration of quinine a great many years ago. If there should be any interest in the matter of priority in recommendation of such a therapeutic approach in malaria, certainly Col. Craig and not ourselves deserves the credit of having first drawn attention to the possible merit of this type of procedure. In passing, however, it might just be of interest to note that possibly the superiority of the method had been attested and forgotten long before Col. Craig introduced it, for Boyd in the A.A.A.S. Symposium on Human Malaria, page 5, 1941, writes as follows: "The earliest report of its employment in the United States that has come to our attention is by Henry Perrine (1826), a physician of Natchez, Mississippi. He employed from six to twelve grains every two to three hours at any period of the fever continued until its symptoms in pulse and skin were subdued, repeating if the return of the fever was suggested."

As a matter of fact, it was not a frequent-dosage method in which we were primarily interested in our work but an attempt to study a continuous method of administration, and it is our hope that a continuous method as advocated in our paper will be given clinical trial with proper controls by those in a position to do so.

Harry Beckman
Jane Smith

The Journal of Laboratory and Clinical Medicine

VOL. 29

AUGUST, 1944

No. 8

CLINICAL AND EXPERIMENTAL

THE CLINICAL USE OF PHTHALYLSULFATHIAZOLE

EDGAR J. POTH, M.D., PH.D., AND CHARLES A. ROSS, M.S.,
GALVESTON, TEXAS

AN EXTENSIVE study covering twenty acylated sulfonamides^{1,2} in an attempt to find substances possessing antibacterial properties and being poorly absorbed from the alimentary tract has resulted in the synthesis and discovery of several compounds fulfilling these specifications. The first of these drugs of therapeutic value, succinylsulfathiazole, was described in 1941 by Poth and Knotts,³ having been synthesized by Miller, Rock and Moore.⁴

This compound when administered by mouth profoundly alters the bacterial flora of the gastrointestinal tract and the physical character of the stools. The use of this drug⁵ in the preoperative preparation of patients due for operations upon the large bowel brought the individuals to operation in excellent physical condition with the bowel emptied of fecal contents and nicely prepared for surgical treatment. The simplification of the bacterial flora of the gastrointestinal tract by the significant reduction of and by the frequent elimination of the coliform organisms, the *Clostridia*, the bacteroides, and numerous other microorganisms offers considerable protection against the development of a fatal peritonitis, especially as emphasized by the studies of Meleny, Olpp, Harvey and Zaytseff-Jern (1932)⁶ who demonstrated that the intraperitoneal inoculation of mixtures of *B. coli*, *B. welchii*, and alpha streptococci was ten to fifteen times as lethal as pure cultures of these organisms. Postoperatively, the patients treated preoperatively with a drug like succinylsulfathiazole do well, there having resulted no instances of peritonitis or locally detectable infection, although operations were often performed on the open colon. These patients experienced a smooth convalescence with minimal gaseous distention and gas pains.

Succinylsulfathiazole has been used for the treatment of bacillary dysentery with excellent results first by Poth, Chenoweth, and Knotts (1942)⁷ and subse-

This work was aided by a grant from Sharp & Dohme, Inc., Philadelphia.
From the Department of Surgery, University of Texas Medical Branch.
Received for publication, March 13, 1944.

quently by numerous investigators. The most significant investigation having been reported by Hardy, Burns, and De Capito⁸ who showed it to be the most effective of the sulfonamides in preventing the development of carriers.

The toxicity of succinylsulfathiazole is particularly low and is probably the least toxic of all the effective sulfonamides which have been used extensively. But, it should be repeatedly emphasized that all members of this series of drugs can be expected to show toxic reactions and that these reactions may indeed be serious if administered to a sensitized or highly susceptible individual. The incidence of the more severe reactions is very low for succinylsulfathiazole. A single instance of agranulocytosis with a fatal outcome has been reported by Johnson (1943).^{9, 10} It is not entirely clear that this complication was due to the action of succinylsulfathiazole although it is true that the individual had previously been shown to have been sensitive to sulfathiazole.

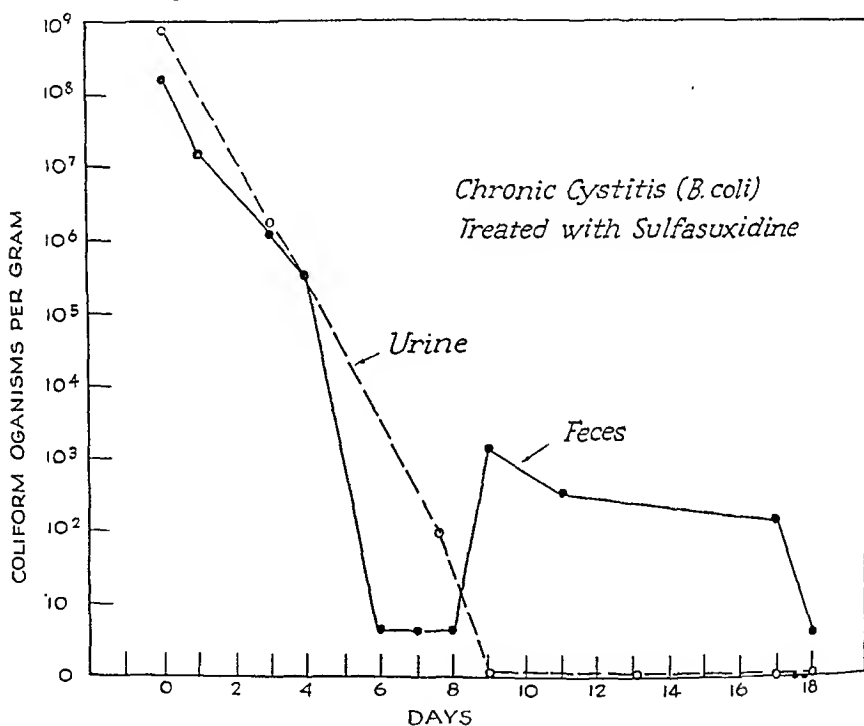


Chart 1—Demonstration of rapid disappearance of *B. coli* from the urine of a patient with a long history of chronic cystitis. The urine became sterile between the third and ninth days. No *B. coli* was present in the feces on the sixth day. The patient received 0.25 Gm. of succinylsulfathiazole daily divided into six equal doses administered orally at four-hour intervals.

The following patient presents a particularly interesting therapeutic application of succinylsulfathiazole. An 82-year-old woman gave a history of abdominal distention and discomfort daily over a period of ten years. She had been troubled with constipation most of her life. There had been recurring attacks of pyelitis and cystitis. When first seen two years ago, she was suffering from an unusually severe urinary tract infection and a moderate anemia and leucopenia were present. There were 1,000,000 *B. coli*, per cubic centimeter in the urine. Under treatment with succinylsulfathiazole, the coliform organisms in the feces and urine were reduced as indicated in Chart 1. The urine became

sterile. The drug was discontinued after three weeks. The constipation and flatulence were relieved. After one month, evidence of a cystitis returned and the urine again contained *B. coli*. Immediate response to succinylsulfathiazole therapy occurred again. After this sequence of events had repeated itself several times and upon the insistence of the patient, and, since the hemoglobin content of the blood had increased and the leucocyte count had improved, 0.125 Gm. of succinylsulfathiazole per kilogram of body weight divided into three daily doses were administered for one week each month. This regimen has kept the patient free of cystitis, flatulence and constipation and no untoward reactions have occurred during a period of two years.

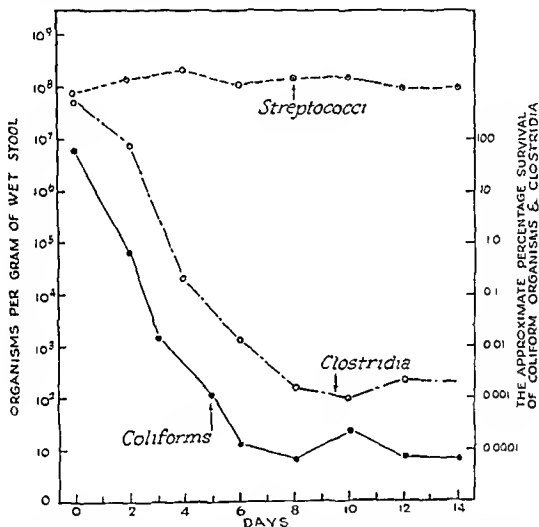


Chart 2—An illustration of the variation of different bacterial groups following the oral administration of 0.0833 Gm. to dogs on a subnormal diet, coliform organisms and for the *Clostridia* representing to record that after 1 resistant spores remain in two dogs. The individual instances.

per kilo of body weight every four hours. The streptococci are not affected. The number in a regular manner. The counts active forms and spores, and it is interesting forms disappear with only the heat-averages of the observations made on the limits of experimental error in all

Poth and Ross (1943)^{2, 11} reported experimental studies on phthalylsulfathiazole as an intestinal antiseptic. This compound, first synthesized by Moore and Miller (1942),¹² was shown to be from two to four times as effective as succinylsulfathiazole in altering the coliform flora in the alimentary tract of the dog. When phthalylsulfathiazole was administered orally to dogs every four hours in doses varying from 0.04 to 0.08 Gm. per kilogram of body weight, the alteration of the coliform flora in the feces was comparable to that observed when 0.166 Gm. of succinylsulfathiazole were similarly administered.

As indicated in Chart 2, phthalylsulfathiazole reduces the *Clostridia* as well as the coliform bacteria to significantly low populations in the feces. It is

interesting that after forty-eight hours all anaerobic vegetative organisms disappear with only heat resistant spores persisting. Similar to the other sulfonamides, phthalylsulfathiazole is ineffective against the alpha *Streptococcus fecalis*. This drug alters the physical character of the stools by reducing the fecal odor, but the feces are rendered less liquid than is the case following the administration of succinylsulfathiazole. This latter finding might be considered an indication that phthalylsulfathiazole would be more effective in the presence of diarrhea, and consequently, be better suited to the treatment of the dysenteries, and be a possible therapeutic agent against cholera and in the treatment of the highly fatal, epidemic, neonatal diarrheas.

Phthalylsulfathiazole is rapidly excreted in the urine of the dog, and the concentration of the drug in the blood has not been observed to exceed 3.0 mg. per 100 cubic centimeters following sustained oral administration of the compound in effective therapeutic doses. No toxic manifestations occurred following such administration of the compound. A single dose of twenty-five Gm. per kilogram of body weight of phthalylsulfathiazole given by stomach tube produced no toxic symptoms, and the concentration of the drug in the blood did not exceed 2.0 mg. per 100 cubic centimeters.

Dogs survived the intravenous administration twice daily of one gram of the sodium salt of phthalylsulfathiazole per kilogram of body weight, for periods of seven days, although vomiting occurred during and following the injections and the animals showed a moderate loss of weight. Intraperitoneal injection of a suspension of the drug is less well tolerated. The L.D. 50 is approximately 2.5 Gm. per kilogram of body weight in single doses intraperitoneally.

THE ADMINISTRATION OF PHTHALYLSULFATHIAZOLE TO MAN

On the basis of the evidence of bacteriostasis and low toxicity of phthalylsulfathiazole obtained by the authors^{2, 11} and by Mattis, Benson & Koelle¹³ on various laboratory animals, the administration of the drug to man was begun in July, 1942. First, the administration was to individuals with normal alimentary tracts and then to persons with various gastrointestinal lesions and disorders including carcinomata of the colon, ulcerative colitis, nonspecific diarrheas, and bacillary dysentery.

When phthalylsulfathiazole is given to individuals with normal gastrointestinal mucosa in doses varying from 0.05 to 0.25 Gm. per kilogram of body weight daily divided into six equal portions administered at four-hour intervals day and night, the alteration of the coliform bacteria occurs as indicated in Chart 3. Doses of phthalylsulfathiazole as small as 0.017 Gm. per kilogram every four hours may effect a significant lowering of the coliform population in the feces of normal individuals. The time required to cause this alteration may be somewhat more prolonged than when larger doses are given. The comparative effectiveness of phthalylsulfathiazole and succinylsulfathiazole is presented in Chart 4 and demonstrates phthalylsulfathiazole to possess, in this instance, twice the bacteriostatic action as that shown by succinylsulfathiazole.

The effect of spacing the frequency of dosage is studied by the daily oral administration of 0.0625, 0.125, and 0.25 Gm. of phthalylsulfathiazole, dividing the respective daily doses, in each instance, into six, three, and single portions and giving the drug at 4, 8, and 24-hour intervals. Charts 5, 6, and 7 show the

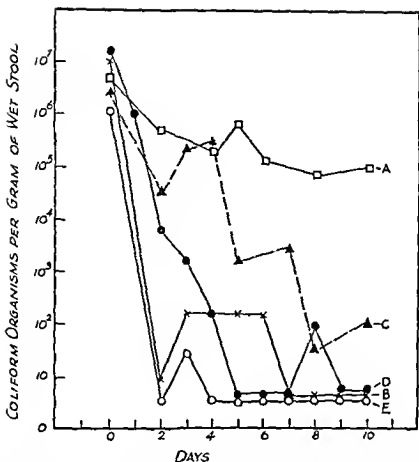


Chart 3.—The effect of various dosages of phthalylsulfathiazole on the coliform bacteria in the gastrointestinal tract of man. A represents the results after the oral administration of 0.05 Gm. per kilogram of body weight daily, divided into six equal portions and given at four-hour intervals; B, 0.0625 Gm. per kilogram; C, 0.10 Gm. per kilogram; D, 0.125 Gm. per kilogram; and E, 0.25 Gm. per kilogram.

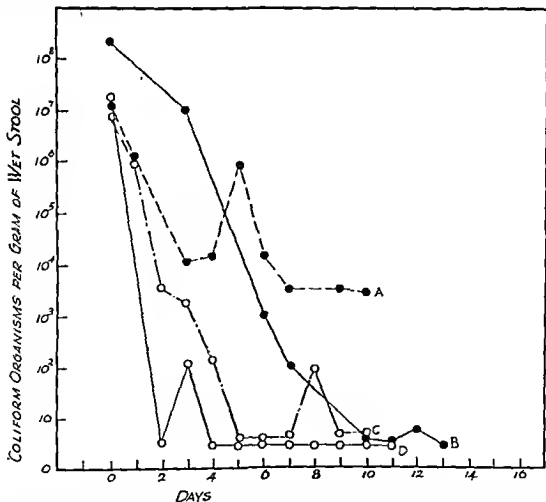


Chart 4.—A comparison of the relative bacteriostatic activity of succinylsulfathiazole and phthalylsulfathiazole as indicated by the alteration of the coliform flora in the gastrointestinal tract of man when equal doses of the drugs are administered orally at four-hour intervals. A represents the results following a daily dosage of 0.125 Gm. of succinylsulfathiazole per kilogram of body weight; B, following a daily dosage of 0.25 Gm. of succinylsulfathiazole; C, following a daily dosage of 0.125 Gm. of phthalylsulfathiazole; and D, following a daily dosage of 0.25 Gm. of phthalylsulfathiazole.
Compare A with C, and B with D.

alteration to be significant in each instance, but the rate of change and the magnitude of the change is decreased as the dosage is lowered and the interval of administration is lengthened. When the smallest dose, however, is given, once daily, the coliform flora is lowered in count from 10^7 to 10^4 , but even this change represents a disappearance of 99.9 per cent of the coliform bacteria initially present. These observations indicate that phthalylsulfathiazole may be better suited to prolonged administration as is required in the treatment of ulcerative colitis, because it need not be given at such frequent intervals. Likewise, it may find use as a prophylactic measure when it is known that an individual is exposed to acute bacillary infections such as dysentery and possibly cholera, as has been suggested previously by Poth, Chenoweth, and Knotts (1942).⁷

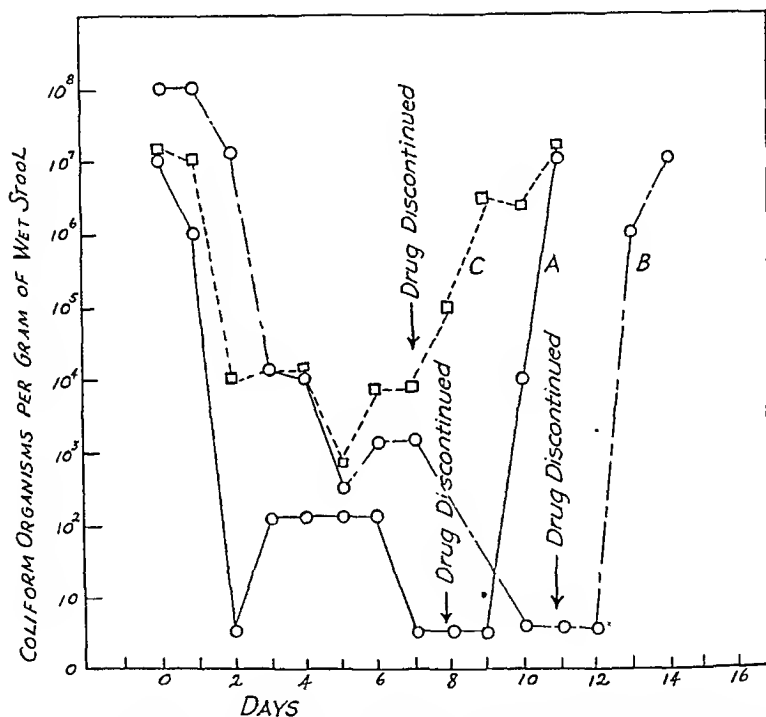


Chart 5.—The effect of spacing the oral dosage of 0.0625 Gm. of phthalylsulfathiazole per kilogram of body weight daily as indicated by the change in the number of coliform bacteria in the gastrointestinal tract of man when the drug is (A) divided into six equal doses and given at four-hour intervals; (B) divided into three equal doses and given at eight-hour intervals; and (C) given as a single daily dose. Compare with Charts 6 and 7.

One of the drawbacks encountered in the administration of succinylsulfathiazole is that the drug tends to be ineffective in the presence of a watery diarrhea. Since the stools do not become as liquid following the administration of phthalylsulfathiazole as when the former drug is given and since phthalylsulfathiazole has the greater bacteriostatic activity, the value of the drug was studied in the presence of a watery diarrhea. An extremely watery diarrhea was produced and maintained for a period of two weeks by the administration of an ounce of magnesium sulfate twice daily to a healthy adult Negro male. Phthalylsulfathiazole, 0.125 Gm. per kilogram of body weight daily, divided

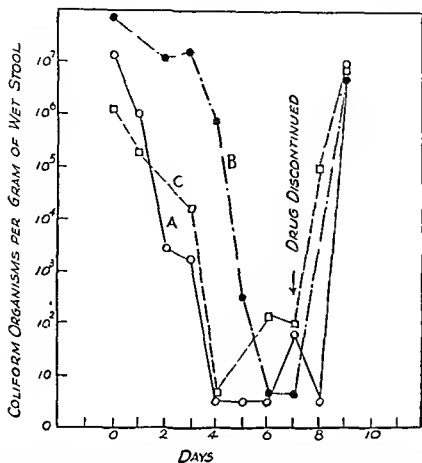


Chart 6.—The effect of spacing the oral dosage of 0.125 Gm. of phthalylsulfathiazole per kilogram of body weight daily, as indicated by the change in the number of coliform bacteria in the gastrointestinal tract of man, when the drug is (A) divided into six equal doses and given at four-hour intervals, (B) divided into three equal doses and given at eight-hour intervals, and (C) given as a single daily dose. Compare with Charts 5 and 7.

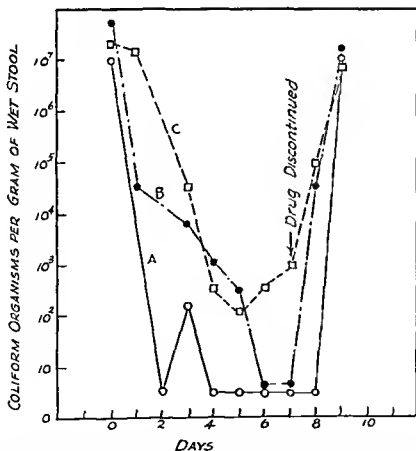


Chart 7.—The effect of spacing the oral dosage of 0.25 Gm. of phthalylsulfathiazole per kilogram of body weight daily was indicated by the change in the number of coliform bacteria in the gastrointestinal tract of man when the drug is (A) divided into six equal doses and given at four-hour intervals, (B) divided into three equal doses and given at eight-hour intervals; and (C) given as a single daily dose. Compare with Charts 5 and 6.

into six equal portions reduced the coliform organisms significantly, but the reduction was not maintained. On doubling the dosage after the tenth day, the coliform count was further reduced and maintained at a low level as indicated in Chart 8. These observations further emphasize the possibility that this compound will be particularly effective therapeutically in the presence of a severe diarrhea and will permit preoperative purgation when deemed necessary to empty the large bowel.

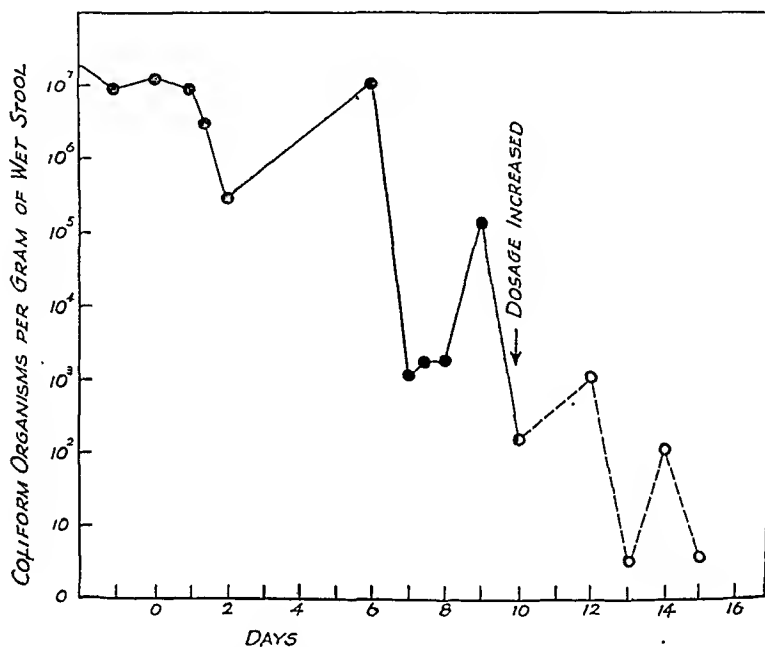


Chart 8.—Illustrating the effect of a watery diarrhea on the efficiency of phthalylsulfathiazole in altering the coliform count in the gastrointestinal tract of man. This patient received an ounce of magnesium sulfate every twelve hours for fifteen days to maintain a watery diarrhea. For the first two days 0.125 Gm. of phthalylsulfathiazole per kilogram of body weight was given by mouth at four-hour intervals as six divided doses. On the tenth day the daily dosage was increased to 0.25 Gm. per kilogram daily.

THE BACTERIOSTATIC ACTIVITY OF PHTHALYLSULFATHIAZOLE

Why should phthalylsulfathiazole have a greater local bacteriostatic activity against coliform organisms in the gastrointestinal tract than succinylsulfathiazole although the solubility of the latter exceeds that of the former compound? At least a partial answer to this question is available. If the two compounds are administered orally in equal doses and the stools are analyzed for both the free and conjugated forms of the drugs, it becomes evident, as is demonstrated by Chart 9, that both the relative and absolute concentrations of the free form of the drugs vary considerably. In the case illustrated the concentration of the free drug, which may or may not be sulfathiazole, coincident with the administration of succinylsulfathiazole attains a concentration of 250 mg. per one hundred Gm. of wet stool, while the corresponding value during the ingestion of phthalylsulfathiazole is 1,250 mg. per cent. At this dosage level, phthalylsulfathiazole is fully effective bacteriostatically against the coliform organisms while this may not be true for succinylsulfathiazole. These data would

indicate that the bacteriostatic activity parallels the concentration of the diazotizable form of the drug in the feces.

An analysis of the urinary excretion of succinylsulfathiazole and phthalylsulfathiazole on 50 successive trials of each gives the following data: (1) when succinylsulfathiazole is administered orally in a dosage of 0.25 Gm. per kilogram of body weight daily in six equal portions, the free form of the drug is excreted to the amount of 0.37 Gm. while 0.49 Gm. of the conjugated form is excreted in 24 hours, or a ratio of 1 to 1.33; (2) when phthalylsulfathiazole is likewise administered in a dosage of 0.125 Gm. per kilogram, the free form of the drug is excreted to the amount of 0.100 Gm. while 0.104 Gm. of the conjugated form is excreted, or a ratio of 1 to 1.04. In conclusion, it is obvious that relatively more of the free form of the drug is excreted when phthalylsulfathiazole is ingested, although definitely a much smaller absolute quantity of the free form of drug is excreted than when succinylsulfathiazole is administered, regardless of the fact that the concentration of the diazotizable form of drug in the feces is roughly five times as great when the phthalyl derivative is given.

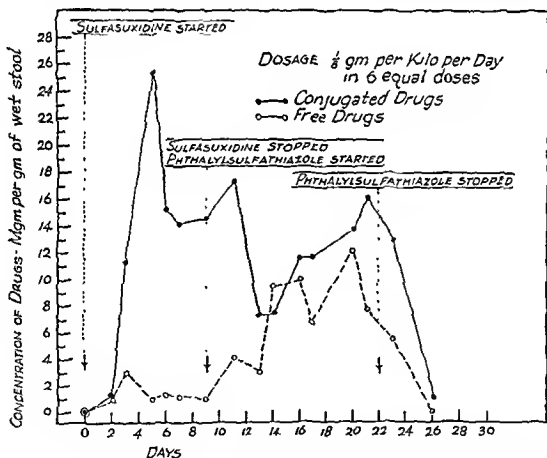


Chart 9.—Comparison of the concentrations of succinylsulfathiazole, phthalylsulfathiazole, and sulfathiazole in the feces after the oral administration of equal quantities of succinylsulfathiazole and phthalylsulfathiazole to man.

A further analysis of the urinary excretion of the free and conjugated forms of phthalylsulfathiazole is given in Chart 10. There is little difference in the quantity of drug excreted in the urine whether a dosage of 0.125 Gm. per kilogram of body weight is ingested as illustrated in Curve B, or 0.065 Gm. per kilogram as shown in Curve C. But, it naturally follows that the percentage excreted in the second instance is roughly twice that of the first example. On the larger dosage as represented by Curve A, the percentage of the ingested drug excreted in the urine is least. If the urinary output of drug is determined in

a large number of cases, it is found that the quantity of drug excreted is essentially constant, which means that, within therapeutic limits, the quantity of drug absorbed from the bowel and excreted in the urine is but little affected by the ingested dose. It can, therefore, be anticipated that larger doses will not be appreciably more toxic than smaller therapeutic doses of phthalylsulfathiazole.

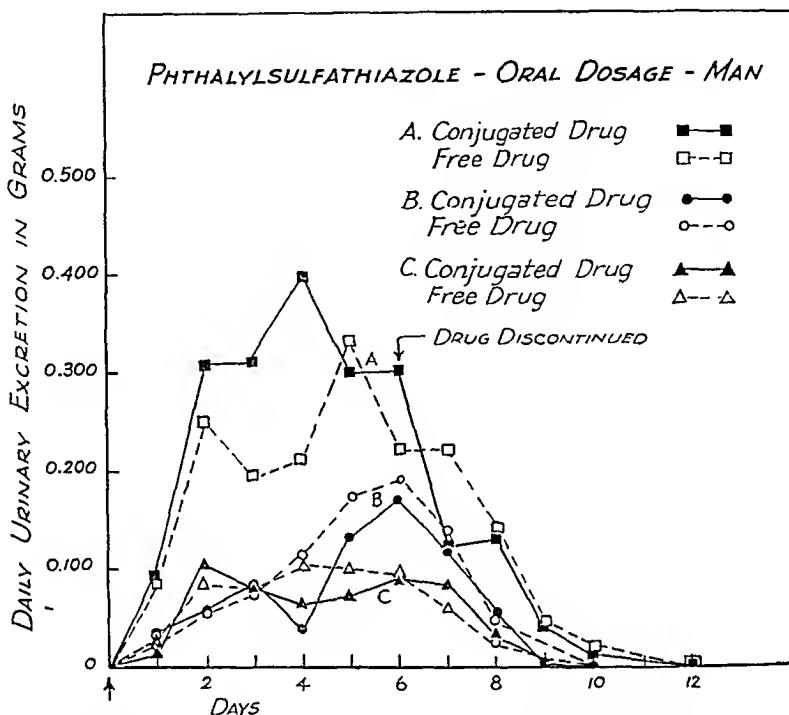


Chart 10.—Absorption and excretion of phthalylsulfathiazole by man.

A. Dosage 0.25 Gm. per kilogram per day in six divided doses at four-hour intervals by mouth; 3.35 per cent of the ingested drug recovered from the urine.

B. Dosage 0.125 Gm. per kilogram per day in six divided doses at four-hour intervals by mouth; 4.48 per cent of the ingested drug recovered from the urine.

C. Dosage 0.0625 Gm. per kilogram per day in six divided doses at four-hour intervals by mouth; 9.2 per cent of the ingested drug recovered from the urine.

An intangible mass of evidence continues to present itself that these compounds, containing free carboxyl groups in their molecules, possess antibacterial properties which are not entirely dependent upon the presence of sulfathiazole presumably resulting from hydrolysis. Although the simplest and most logical assumption would be that all the effect is contributed by sulfathiazole, it seems highly probable that the physical and chemical properties of these compounds are operative in determining their ultimate, local bacteriostatic activity by influencing the absorption and adsorption of these relatively inactive acylated compounds by the individual bacterium.

THE VALUE OF SULFATHIAZOLE AS AN INTESTINAL ANTISEPTIC

In 1942, Poth, Knotts, Lee and Inui¹ showed that sulfanilamide, sulfadiazine, and sulfathiazole had little bacteriostatic effect in the alimentary tract of the dog as demonstrated by the alteration of the coliform organisms.

Because repeated reports have suggested that sulfathiazole and sulfadiazine have sufficient bacteriostatic activity locally in the bowel to sterilize the feces (Eisenhoff and Goldstein, 1943),¹⁴ an additional study was made on human subjects receiving sulfathiazole in relatively large doses. Representative data are given in Chart 11. In the majority of instances, there is no significant alteration of even the coliform organisms although large doses of sulfathiazole were administered. A total of 56 twenty-four-hour collections of urine were analyzed and showed that 36 to 76 per cent, or an average of 51 per cent, of the ingested sulfathiazole was excreted in the urine. The blood levels varied from 3.4 to 7.6 mg. per cent. Assays of stools for the drug gave a concentration of 80 to 170 mg. per cent.

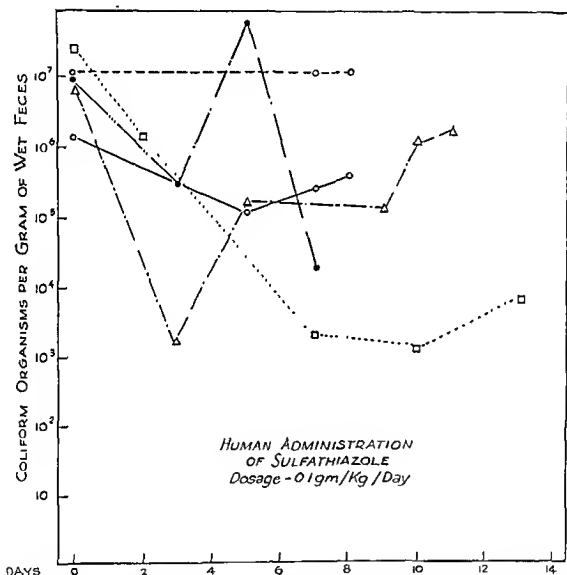


Chart 11.—A plot of the alteration of the coliform flora in the normal gastroenteric tract of man following the oral administration of full therapeutic doses of sulfathiazole. As will be noted, there may be a significant alteration in the number of coliform organisms, but this change is neither consistent nor always maintained.

In a series of several thousand stool cultures over a period of four years, we have never encountered a single instance following the administration of either succinylsulfathiazole or phthalylsulfathiazole in which the feces were sterile. It is not, however, unusual to find no coliform bacteria, and it should not be expected to find the feces free of the alpha *Streptococcus fecalis*, since the growth of this normal inhabitant of the alimentary tract is not significantly influenced by the sulfonamides. The *Shigella paradysenteriae* organisms are highly susceptible to the bacteriostatic action of the sulfonamides and show a

good therapeutic response to sulfapyridine, sulfathiazole and sulfadiazine even though it is not possible to maintain as high a concentration of these drugs in the alimentary tract as is possible when succinylsulfathiazole or phthalylsulfathiazole are administered.

THE TREATMENT OF DIARRHEAS WITH PHTHALYLSULFATHIAZOLE

Phthalylsulfathiazole has proved to be highly specific in the treatment of (Flexner) bacillary dysentery (Poth, 1943).¹⁵ This therapeutic result could be anticipated from the experimental observations that the drug is quite effective in inhibiting the growth of coliform bacteria in the presence of a watery diarrhea (Chart 8), and that a relatively high concentration of a diazotizable derivative is maintained in the feces.

It has, furthermore, been observed that patients suffering from diarrheas, but in whom the *Shigella paradysenteriae* organisms could not be identified, frequently respond promptly following the administration of phthalylsulfathiazole. Ordinarily, the most satisfactory therapeutic regimen is to give 0.04 Gm. of the drug per kilogram of body weight every four hours for 12 doses and then 0.02 Gm. per kilogram at 4-hour intervals until diarrhea ceases or for a total

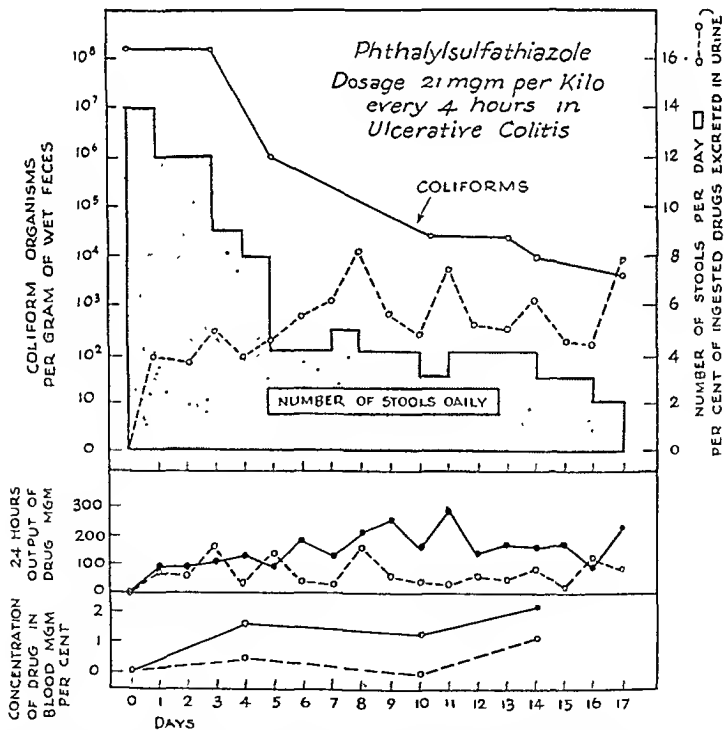


Chart 12.—The data plotted in this chart is that of D. L. B. listed in Table I. After an illness of five years' duration with practically continuous diarrhea, this patient entered the John Sealy Hospital with an abscess in right flank, which drained spontaneously and was demonstrated to be perineal. History revealed that the abscess had been drained two years previously, and that the patient had had a daily average of 14 bloody, mucus-containing stools. Ameba and cysts could not be demonstrated in the feces. After five days' administration of phthalylsulfathiazole, the number of stools decreased to four, and they changed markedly in character. They were becoming formed and the blood and mucus practically disappeared. Subsequently the stools became odorless, formed, and of normal appearance. The patient had only one or two movements daily. The draining abscess healed. It was irrigated with azo-chloramide three times daily. While the chart covers 17 days, this patient received treatment for 12 weeks without evidence of toxicity and with continued improvement and weight gain. Patient taking a liberal bland diet.

of 7 days if the diarrhea is proved to be a bacillary dysentery. The feces should be negative for *Shigella paradysenteriae* on three weekly cultures following treatment if carriers are to be eliminated. Only rarely are positive stools found on follow-up cultures, and, in these instances, the individuals should be subjected to 10 days' additional therapy with 0.02 Gm. of phthalylsulfathiazole per kilogram of body weight every four hours and be observed again by culture. It is not necessarily true that the action of either succinylsulfathiazole or phthalylsulfathiazole is entirely due to sulfathiazole resulting from a simple hydrolysis of these acylated compounds. These condensation products may possess in themselves intrinsic properties leading directly or indirectly to manifested antibacterial activity.

THE TREATMENT OF ULCERATIVE COLITIS WITH PHTHALYLSULFATHIAZOLE

The treatment of ulcerative colitis will, of course, be determined by the underlying cause whenever it is possible to establish such an etiological factor. Since amebiasis may be the etiological basis of ulcerative colitis, all patients having this disease complex should receive adequate treatment with one of the amebicidal drugs even though no evidence of amebiasis can be demonstrated.

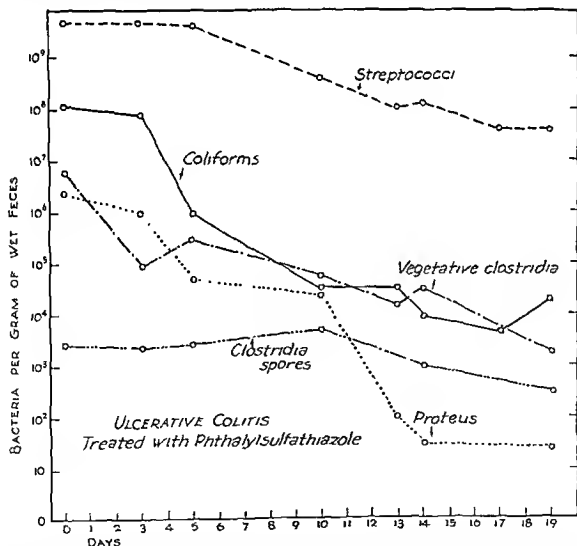


Chart 13.—A plot for the complete cultural data on D. L. B. tabulated in Table I for the initial 19 days of therapy. The rate of alteration of the bacterial flora in this patient is slower than usual because of the extensive ulcerative changes in the bowel. The entire colon has been converted to an extremely narrow tube. The streptococci are unaffected. The coliform organisms and the vegetative *Clostridia* are progressively decreasing in number. The spores of *Clostridia* have not been altered. *B. proteus* has largely disappeared. It is interesting to note that this patient had experienced considerable clinical benefit by the fifth day of therapy before there was a demonstrable change in the bacterial flora (see Chart 12). The ultimate and proper treatment will, no doubt, be colectomy, and the patient is being prepared accordingly.

Six cases of chronic ulcerative colitis have been treated with phthalylsulfathiazole. The clinical response has been satisfactory in each instance. During the initial period of four weeks, an oral dosage of 0.02 Gm. per kilogram of body weight was given every four hours. The usual early course is summarized in Charts 12 and 13.

One patient has been treated continuously for ten months with phthalylsulfathiazole without evidence of toxic drug reactions. During this period a multiple fistula in ano has healed and remained healed for three months, the bowel has regained much of its normal architecture, and the patient has had a severe upper respiratory infection without recurrence of the diarrhea which has been reduced from 10 stools containing blood, pus, and mucus to a single daily formed passage.

The number of cases treated is too small to draw any definite conclusions, but it appears that this compound is at least as effective as any of the other sulfonamides in bringing about and maintaining the patient in a remission. It deserves further study.

PHTHALYLSULFATHIAZOLE AS AN ADJUVANT IN SURGERY OF THE COLON

Succinylsulfathiazole definitely alters the postoperative course of patients upon whom operations on the colon are performed. The regimen used during the preoperative and postoperative period was outlined by us in 1942. It has been demonstrated repeatedly that such patients experience a smooth postoperative course (Allen, 1943,¹⁶ and Behrend, 1944²¹).

Phthalylsulfathiazole may, likewise, be used. Its administration may be advantageous in those instances where the patient has a diarrhea such as is encountered, not infrequently, in the presence of malignancies of the cecum and ascending colon, as well as in those cases when it is desirable to resort to purgation to clear the bowel.

The ordinary course of preoperative preparation includes early purgation when necessary, a low residue diet or even a completely residue-free diet such as may be obtained when an amino acid-carbohydrate mixture such as "Nutramigen" is given, the administration of 0.02 Gm. of phthalylsulfathiazole per kilogram of body weight every four hours, and observation of the odor, consistency and bacterial count of the stools. As an indication of the alteration of the bacterial flora, the change in the coliform count is readily followed by the streak plate technique previously described in 1942.¹⁷

A small representative group of patients is presented in detail in Table I. Approximately five per cent of the ingested drug is excreted in the urine. In view of the smaller dosage of phthalylsulfathiazole as compared to that of succinylsulfathiazole only about half the absolute quantity of phthalylsulfathiazole is excreted by the kidneys.

Qualitatively the comparative effects of phthalylsulfathiazole and succinylsulfathiazole are quite similar, while quantitatively the former drug has approximately twice the antibacterial activity.

TOXICITY OF PHTHALYLSULFATHIAZOLE

There have been few toxic drug reactions seen following the oral administration of phthalylsulfathiazole. From the fact that this compound is readily

hydrolyzed in acid solution, it should follow that toxic reactions would occur more frequently than when succinylsulfathiazole is given, especially when it is observed that much higher concentrations of free drug are maintained in the colon when phthallylsulfathiazole is administered. This supposition has not been substantiated by the observed facts.

A single moderately severe toxic reaction has been observed in a 61-year-old woman with a large, redundant colon which was otherwise normal excepting for a small rectal polyp showing early malignancy. Within 24 hours after receiving 0.02 Gm. of phthallylsulfathiazole per kilogram of body weight every four hours, this patient complained of a generalized aching accompanied by headache, followed by nausea, and a chilling sensation within an additional twelve hours. A swinging type of fever developed to reach a maximum of 104.4° F. orally on the fifth day of therapy. While the patient continued to be nauseated for the entire period of drug administration, she did not vomit. The maximum concentration of the drug in the blood did not exceed 2.5 mg. per 100 cubic centimeters. The coliform organisms dropped from 10^7 to 10^2 bacteria per gram of wet stool during the five days of drug therapy. Crystalluria did not occur. No red cells appeared in the urine and there were no cytological changes in the blood. Subsequently, this patient received 0.04 Gm. of succinylsulfathiazole per kilogram of body weight every four hours, and again similar symptoms developed with nausea, headache, malaise, and a temperature elevation to 102° F. within 48 hours of administration. Obviously, this patient is sensitive to the sulfonamides.

A stoichiometric analysis of the average therapeutic dosages of sulfathiazole, succinylsulfathiazole and phthallylsulfathiazole is illustrated in Table II. The millimolar equivalent dosage of phthallylsulfathiazole is about 40 per cent that of succinylsulfathiazole and is approximately equal to that of sulfathiazole. The difference in the equivalent molecular quantities excreted in the urine is strikingly demonstrated. Fifteen times as many molecular equivalents of sulfathiazole are excreted as is the case with phthallylsulfathiazole.

Crystalluria has not been observed to follow the oral administration of phthallylsulfathiazole. The absence of crystals of drug in the urine is due to the facts that the free form of the drug is not excreted in large quantities and because the conjugated drug forms soluble salts even at a pH of 5.6.

As an increasing number of patients receive this drug, it can be expected to encounter the usual toxic manifestations of the sulfonamides, but it can be predicted that such reactions will be relatively infrequent. It must again be emphasized that any patient receiving this drug should be under constant observation.

DISCUSSION

The investigation of these various acylated sulfonamides has introduced a series of compounds which have properties such that they tend to limit their action locally to the alimentary tract because they are sparingly absorbed from the bowel. These compounds are characterized by having a free carboxyl group in the molecule and form readily soluble and highly ionized salts. It is, therefore, possible to maintain exceedingly high concentrations of these sulfonamides in the gastrointestinal tract without untoward toxic manifestations on the

TABLE I

THE DOSE OF PHTHALYLSULFATHIAZOLE WITH THE CONCENTRATION OF THE DRUG IN THE BLOOD AND THE FECES AND THE QUANTITY OF "FREE" AND "CONJUGATED" DRUG EXCRETED IN THE URINE

Patient and Weight in Kg.	Day	Daily Dose in Gm.	Number Doses Daily	FREE CONJUGATED		Concentration of Drug in Blood	FREE CONJUGATED		Daily Urinary Output in Gm.	FREE CONJUGATED		Concentration of Drug in Stools	Mg.s./Gm.	COMMENTS
				FREE	CONJUGATED		FREE	CONJUGATED		FREE	CONJUGATED			
A. B. 45	1	6	6	--	--	--	--	--	--	--	--	--	--	A healthy 26-year-old white female with normal gastroenteric canal used to study the effect of 0.125 Gm. phthalylsulfathiazole per kilo of body weight daily when divided into 6 doses, 3 doses, and 1 dose, taken at 4, 8, and 24-hour intervals, respectively. The alteration of the coliform count is presented in Chart 6. No toxic drug reaction occurred during the 49 days' administration. Given at 4-hour intervals 4.86% of ingested drug was excreted in the urine.
	2	6	6	--	--	--	0.026	0.032	0.026	0.032	4.2	17.6		
	3	6	6	0.1	0.2	0.2	0.055	0.058	0.055	0.058				
	4	6	6	0.6	0.2	0.2	0.077	0.087	0.077	0.087				
	5	6	6				0.113	0.039	0.113	0.039				
	6	6	6				0.175	0.135	0.175	0.135	2.4	7.3		
	7	6	6	0.3	0.2	0.2	0.180	0.150	0.180	0.150	9.6	7.3		
	8	6	6				0.192	0.172	0.192	0.172	10.0	11.5		
	9			0.4	0.4	0.4	0.140	0.125	0.140	0.125	6.4	11.6		
	10						0.050	0.056	0.050	0.056				
	11			0.3	0.4	0.4	0.028	0.000	0.028	0.000	4.0	2.8		
	12						0.007	0.005	0.007	0.005				
	13	6	6				0.005	0.004	0.005	0.004				
	14	6	6	0.6	0.0	0.0	0.016	0.022	0.016	0.022	0.9	4.4		
	15	6	6				0.057	0.072	0.057	0.072				
	16	6	6	0.6	0.0	0.0	0.100	0.075	0.100	0.075				
	17	6	6				0.105	0.077	0.105	0.077	12.3	13.9		
	18	6	6	0.4	0.0	0.0	0.119	0.092	0.119	0.092				
	19						0.084	0.086	0.084	0.086				
	20						0.120	0.104	0.120	0.104	7.8	16.0		
	21			0.1	0.2	0.2	0.060	0.058	0.060	0.058	5.7	13.0		
	22						0.035	0.032	0.035	0.032				
	23			0.4	0.2	0.2	0.017	0.003	0.017	0.003	0.0	0.0		
	24						0.009	0.000	0.009	0.000				
	25			0.0	0.1	0.1								

Given at 4-hour intervals 4.86% of ingested drug was excreted in the urine.

TABLE I—CONT'D

Patient and Weight in Kg.	Day	Daily Dose in Gm.	Number Doses Daily	Concentration of Drug in Blood		Daily Urinary Output in Gm.		Concentration of Drug in Stools		COMMENTS
				FREE	CONJUGATED	FREE	CONJUGATED	FREE	CONJUGATED	
O. G. 52	1	12	6			0.027	0.110			57-year-old colored male; squamous carcinoma of anus and rectum; patient had no bowel movements without castor oil; coliform count reduced to zero; at operation bowel was clean and odorless; no toxic drug reactions. 5.47 per cent of ingested drug excreted in urine.
	2	12	6	0.4						
	3	6	6	0.4						
	4	6	6			0.240	0.310			
	5	6	6	0.4		0.122	0.450			
	6	6	6			0.103	0.172			
	7	6	6	0.0		0.090	0.123			
	8	6	6			0.079	0.101	8.6	28.6	
	9	6	6	0.4		0.273	0.546			
	10	6	6			0.069	0.098			
	11	6	6	0.4		0.075	0.109			
	12	6	6			0.177	0.190	6.7	10.9	
	13	6	6	0.2		0.128	0.156			
	14	6	6			0.137	0.232			
	15	6	6			0.214	0.210			
	16	patient expired								

28	6	0	0.090	0.062	
29	6	0.4	0.131	0.140	4.9
30	6	0.1	0.103	0.107	6.6
31	6		0.128	0.160	3.9
32	6		0.150	0.175	2.2
33	6	0.4	0.176	0.170	2.1
34	6		0.218	0.281	6.8
35	6	0.6	0.160	0.193	
36	6	0.7	0.168	0.259	
37	6	0.2	0.105	0.116	5.1
38	6	0.4	0.080	0.117	4.0
39	6	0.8	0.095	0.102	
40	6	0.0	0.100	0.120	
41	6	0.2	0.196	0.243	4.2
42	6	0.0	0.100	0.100	5.1
43	6	0.1	0.045	0.032	
44	6	0.0	0.031	0.000	
45	6		0.005	0.000	
46	6	0.0	0.004	0.000	
D. L. B.					
52	0		0.089	0.098	
1	6		0.099	0.071	
2	6		0.103	0.158	
3	0		0.149	0.000	
4	0	1.6	0.089	0.137	
5	0		0.190	0.028	
6	0		0.143	0.000	
7	6		0.226	0.167	
8	6		0.168	0.063	
9	6		0.155	0.038	
10	6	1.4	0.290	0.000	
11	6		0.140	0.065	
12	6		0.185	0.043	
13	6		0.163	0.088	
14	6	2.2	0.168	0.000	
15	6		0.092	0.098	
16	6		0.240	0.091	
17	6				

30-year-old male; history of chronic ulcerative colitis of long standing; diarrhea for past 5 years; received sulfanylgundine about 2 years ago and suffered severe toxic reaction without alteration of diarrhea. For alteration of flora and diarrhea upon administration of phthalylsulfathiazole see Charts 12 and 13. No toxic reaction to phthalylsulfathiazole. 5.4% of ingested drug excreted in the urine.

30-year-old male; history of chronic ulcerative colitis of long standing; diarrhea for past 5 years; received sulfamylguanidine about 2 years ago and suffered severe toxic reaction without alteration of diarrhea. For alteration of flora and diarrhea upon administration of phthalylsulfathiazole see Charts 12 and 13. No toxic reaction to phthalylsulfathiazole. 5.4% of ingested drug excreted in the urine.

TABLE II

THE COMPARATIVE MILLIMOLAR DOSAGE AND URINARY EXCRETION OF SULFATHIAZOLE, SUCCINYLSULFATHIAZOLE, AND PHTHALYLSULFATHIAZOLE BY MAN

AVERAGE THERAPEUTIC DOSE	EQUIVALENT DOSAGE IN MILLIMOLES	AVERAGE URINARY EXCRETION EXPRESSED IN MILLI-EQUIVA- LENTS PER KILO OF BODY WEIGHT
Sulfathiazole 0.10 Gm./kilo/day (Molecular weight 255)	0.392	0.235 ¹
Succinylsulfathiazole 0.25 Gm./kilo/day (Molecular weight 355)	0.704	0.035 ²
Phthalylsulfathiazole 0.125 Gm./kilo/day (Molecular weight 403)	0.310	0.0155 ²

¹Assuming 60 per cent of the ingested drug to be excreted in the urine. (Barlow and Climenko, 1941.)^{1b}

²Assuming 5 per cent of the ingested drug to be excreted in the urine.

part of the host. The local bacteriostatic action may arise from several sources of which the following immediately present themselves: (1) the intrinsic action of the drug per se and its anion conditioned by its physical and chemical properties to influence the local concentration of the compound within and upon the individual microorganisms dependent upon the specific absorption and adsorption prevailing; (2) the simple hydrolysis of the conjugated drugs to yield the so-called "free" sulfonamides; (3) the formation of compounds in a nascent or excited state by the action of the microorganisms upon the absorbed or adsorbed acylated derivatives to yield substances possessing apparent specific activity merely because the thermodynamic environment is such as to favor the formation of an active compound in a relatively high concentration in the immediate vicinity of or within the cell of the susceptible microorganisms; and, (4) the influence of split products other than the sulfonamide radical; i.e., succinic acid from succinylsulfathiazole. Succinate is of importance in the substrate concerned in certain oxidation-reduction chains involved in respiratory enzyme systems. Such a process may alter the normal metabolism of the cell or even tend to eliminate or prevent the formation of substances which inhibit the more specific competition of the sulfonamides to fix an hypothetical substance required as a normal metabolite by the organisms.

It is not clear that the free or diazotizable form of compound present in the feces following the administration of either phthalylsulfathiazole or succinylsulfathiazole is sulfathiazole or even some other single compound, because the quantity of diazotizable substance excreted in the urine following phthalylsulfathiazole administration is less than one-half the quantity following the administration of succinylsulfathiazole although in the former instance, a diazotizable substance is present in the feces in five times the concentration as is the case during succinylsulfathiazole administration.

It is logical, therefore, to anticipate that these drugs may exhibit individual bacteriostatic properties dissimilar to each other as well as to sulfathiazole. Such dissimilarities may permit one to hope that various of the condensation products might possess individual properties giving these compounds a degree of specificity against different species and strains of bacteria.

Regardless of any theoretical consideration of mechanisms as to action, the fact remains that these drugs induce profound alterations in the bacterial flora of the alimentary tract reducing certain organisms to a very small percentage of the initial population while other organisms appear completely resistant. Because of the fact that bowel contents are not rendered sterile, but rather that the bacterial flora is merely simplified, the value of inducing such an alteration in the preoperative preparation of the colon has been questioned. Meleny and his co-workers⁶ have shown that mixtures of certain bacteria are roughly 15 times as virulent as their pure strains. It has been demonstrated by Poth, Knotts, Lee, and Inui¹ and confirmed by Saltzstein¹⁰ that succinyl-sulfathiazole can so modify the contents of the large bowel of the dog that gross drainage of the descending colon into the peritoneal cavity does not result in fatal peritonitis. Sarnoff and Poth²⁰ have shown that dogs can be protected against necrosis of segments of the ileum following ligation of the venous return by adequately treating the animals with succinylsulfathiazole prior to the ligation.

If the bacteriostatic activity of phthalylsulfathiazole as regards coliform organisms can be taken as an index of the relative antibacterial activities of phthalylsulfathiazole and succinylsulfathiazole for all bacteria, then one can predict that the former possesses roughly twice the activity of the latter compound. This relationship will not, however, be true if either of these compounds or their degradation products have any specificity for any species, type or strain of organisms.

CONCLUSIONS

Phthalylsulfathiazole, a condensation product of sulfathiazole and phthalic anhydride, is an antibacterial agent of considerable interest and of therapeutic possibilities where activity restricted to the alimentary tract is desired. Approximately five per cent of the orally administered therapeutic dose is excreted in the urine. Ordinarily, the concentration of the drug in the blood does not exceed 1.5 mg. per 100 cubic centimeters. As compared to their respective bacteriostatic activities, when measured by their ability to suppress the coliform organisms, phthalylsulfathiazole possesses roughly twice the activity of succinylsulfathiazole. In the absence of diarrhea and ulcerated lesions in the bowel, a single daily dose of phthalylsulfathiazole will effectively lower the coliform organisms in the feces.

The vegetative forms of the *Clostridia* are greatly reduced following the oral administration of phthalylsulfathiazole, and stools are rendered essentially odorless without ordinarily producing a diarrhea. The drug is shown, likewise, to be an effective bacteriostatic agent locally in the bowel as is indicated by the alteration of the coliform bacteria in the presence of a watery diarrhea.

An extensive study of absorption and excretion has shown that an average of 5 per cent of the oral, therapeutic dose of phthalylsulfathiazole is excreted in the urine. Analyses of stools reveal that the content of phthalylsulfathiazole and a "free" diazotizable degradation product chemically similar to sulfathiazole varies between wide limits and that this "free" compound may maintain a concentration of 1,250 mg. per cent.

Preliminary trials of phthalylsulfathiazole in nonspecific diarrheas, bacillary dysentery, chronic ulcerative colitis, and for the preoperative preparation of the large bowel are presented. The drug appears particularly well tolerated by patients having ulcerative colitis and is quite effective in inducing and maintaining prolonged remissions of the disease. Severe toxic manifestations have not been encountered in ulcerative colitis patients even though the therapy has continued for several months. Phthalylsulfathiazole can be maintained in high concentration in the diseased alimentary tract of man with low concentrations of the drug in the blood.

Some of the possible mechanisms through which the acylated sulfonamides act are discussed, and it is proposed that the action of succinylsulfathiazole and phthalylsulfathiazole may not be due either wholly or in part to the formation of sulfathiazole by simple hydrolysis.

As indicated by the alteration of the coliform flora in the bowel of man, phthalylsulfathiazole, *in half the dosage*, is as effective as succinylsulfathiazole.

REFERENCES

1. Poth, E. J., Knotts, F. L., Lee, J. T., and Inui, F.: Bacteriostatic Properties of Sulfanilamide and Some of Its Derivatives. I. Succinylsulfathiazole, a New Chemotherapeutic Agent Locally Active in the Gastrointestinal Tract, *Arch. Surg.* 44: 187, 1942.
2. Poth, E. J., and Ross, C. A.: Bacteriostatic Properties of Sulfanilamide and Some of Its Derivatives. II. Phthalylsulfathiazole, a New Chemotherapeutic Agent Locally Active in the Gastrointestinal Tract, *Texas Rep. Biol. & Med.* 1: 1943.
3. Poth, E. J., and Knotts, F. L.: Succinylsulfathiazole, a New Bacteriostatic Agent Locally Active in the Gastrointestinal Tract, *Proc. Soc. Exper. Biol. & Med.* 48: 129, 1941.
4. Miller, E., Rock, H. J., and Moore, M. L.: Substituted Sulfanilamides: I. N⁴-Acyl Derivatives, *J. Am. Chem. Soc.* 61: 1198, 1939.
5. Poth, E. J., and Knotts, F. L.: Clinical Use of Succinylsulfathiazole, *Arch. Surg.* 44: 208, 1942.
6. Meleny, F. L., Olpp, J., Harvey, H. D., and Zaytseff-Jern, H.: Peritonitis, Synergism of Bacteria Commonly Found in Peritoneal Exudates, *Arch. Surg.* 25: 709, 1932.
7. Poth, E. J., Chenoweth, B. M., and Knotts, F. L.: A Preliminary Report on the Treatment of Bacillary Dysentery With Succinylsulfathiazole, *J. LAB. & CLIN. MED.* 28: 162, 1942.
8. Hardy, A. V., Burns, W., and De Capito, T.: Cultural Observations on the Relative Efficacy of Sulfonamides in *Shigella Dysenteriae* Infections, *Pub. Health Rep.* 58: 689, 1943.
9. Johnson, S. A. M.: Acute Agranulocytosis Due to Administration of Succinylsulfathiazole, *J. A. M. A.* 122: 668, 1943.
10. Poth, E. J., and Johnson, S. A. M.: Correspondence in Regard Granulocytopenia After Use of Succinylsulfathiazole, *J. A. M. A.* 123: 112, 1943.
11. Poth, E. J., and Ross, C. A.: Phthalylsulfathiazole, a New Bacteriostatic Agent, *Fed. Proc.* 2: 89, 1943.
12. Moore, M. L., and Miller, C. S.: Dicarboxylic Acid Derivatives of Sulfonamides, *J. Am. Chem. Soc.* 64: 1573, 1942.
13. Mattis, P. A., Benson, W. M., and Koelle, E. S.: Toxicological Studies of Phthalylsulfathiazole, *J. Pharmacol. & Exper. Therap.* 81: 116, 1944.
14. Eisenhoff, H. M., and Goldstein, H.: The Control of an Outbreak of Bacillary Dysentery With Sulfonamides, *J. A. M. A.* 123: 624, 1943.
15. Poth, E. J.: The Use of Succinylsulfathiazole and Phthalylsulfathiazole as Intestinal Antiseptics, *Texas State J. Med.* 39: 369, 1943.
16. Allen, A. W.: Carcinoma of the Colon, *Surgery* 14: 350, 1943.
17. Poth, E. J.: Succinylsulfathiazole; An Adjuvant in Surgery of the Large Bowel, *J. A. M. A.* 120: 265, 1942.
18. Barlow, O. W., and Climenko, D. R.: Studies on the Pharmacology of Sulfapyridine and Sulfathiazole, *J. A. M. A.* 116: 282, 1941.
19. Saltzstein, H. C.: Discussion of Paper: Poth, E. J.: Succinylsulfathiazole; an Adjuvant in Surgery of the Large Bowel, *J. A. M. A.* 120: 265, 1942.
20. Sarnoff, S., and Poth, E. J.: Unpublished Data.
21. Behrend, M.: Succinylsulfathiazole (Sulfasuxidine) and the Elimination of the Mikulicz Operation, *S. Clin. North America* 24: 238, 1944.

THE ACUTE TOXICITY OF COMMERCIAL PENICILLIN

HENRY WELCH, CLIFFORD W. PRICE, JEAN K. NIELSEN, AND
ALBERT C. HUNTER, WASHINGTON, D. C.

THE large volume of clinical data collected during the relatively short time that penicillin has been used presents extremely strong evidence of the innocuous nature of this drug. The clinical evidence has been obtained on a variety of diseases through the efforts of the Office of Scientific Research and Development in carefully organized clinical studies. Such an accomplishment in normal times would have required years of effort by individual investigators. From the original studies of Chain, et al.¹ and Abraham, et al.,² it has been apparent that even with relatively crude preparations there was little to indicate any contraindication to its use in man. Later studies by Hobby, et al.,³ Herrell, et al.,⁴ Blake and Craige,⁶ and Rammelkamp and Keefer,⁷ have all substantially confirmed the fact that at least so far as penicillin sodium is concerned, little, if any, toxicity can be ascribed to this therapeutic agent. It is true that recently Lyons⁸ has reported an occasional, at present unexplained, sensitization but such a "toxic" effect, if it may be so described, is apparently associated with individual idiosyncrasy rather than with penicillin itself. As pointed out by Lyons, "No significantly harmful effects have been observed."

In testing commercial penicillin for "toxicity" a method has been used which involves the intravenous injection of mice with 100,000 units per kilogram of body weight. Five mice are injected and the product is considered satisfactory if none dies within a forty-eight-hour period of observation. In carrying out routine tests of this nature it soon became obvious that such a test does not measure the "toxicity" of penicillin but rather constitutes a safety test which is capable of eliminating only those products seriously contaminated with substances toxic for mice. In the assay of over 300 lots of penicillin sodium produced for clinical use by fourteen different manufacturers none has failed to pass the safety test. Similarly, commercially produced penicillin calcium and penicillin ammonium were found to be satisfactory when tested by the above method. A commercial preparation of penicillin magnesium, however, failed to pass the safety test, but in this instance the toxicity, as described later in this report, was shown to be due to the cation rather than to penicillin itself.

In order to determine the acute toxicity of commercial penicillin sodium, the material remaining from each lot after assay, which includes tests for sterility, potency, moisture content, safety, and pyrogens, was injected intravenously into mice in increasing dosages. In some instances it was not possible to establish the "toxic level" of units with individual lots because of lack of material. However, it was possible to establish a "pattern" of the acute toxicity of each manufacturer's product by studying several lots, either indi-

Food and Drug Administration, Washington, D. C.
Received for publication, March 14, 1944.

vidually or pooled. At least three mice were injected at each level of units tested and repeat tests were made at the dose found to be lethal to the extent of the material available for testing.

TABLE I
ACUTE TOXICITY OF PENICILLIN SODIUM IN MICE

MANUFACTURER	NO. OF LOTS TESTED	TOXIC DOSE UNITS/20-GRAM MOUSE*	APPROXIMATE RANGE UNITS/MG.
A	12	3,500- 4,500	100-275
B	6	5,000- 8,000	46-120
C	11	7,000- 9,000	130-320
D	7	8,000-18,000	100-346
E	13	10,000-16,000	140-750
F	8	10,000-16,000	140-330
G	7	12,000-14,000	300-400
H	11†	15,000-18,000	200-500
	5†	8,500- 9,500	134-160
I	15	13,000-21,000	250-540
J	12	25,000-32,000	300-500

*All mice observed for 48 hours.

†Recent lots of this manufacturer's product have shown a lower potency and correspondingly higher toxicity.

In Table I are given the number of units of penicillin sodium which consistently caused death of 20-gram mice when injected intravenously. There is a marked difference in the number of units found to be invariably lethal for mice with the various manufacturers' products. The product of manufacturer A in a study of 12 lots consistently killed 20-gram mice in doses of from 3,500 to 4,500 units, while the product of J consistently killed mice at 25,000- to 32,000-unit dosages. It is of interest that an occasional lot of manufacturer J's product failed to kill mice when injected in 30,000- to 35,000-unit doses in 20-gram mice, while manufacturer A's product has never been found to be innocuous in doses greater than 4,500 units per 20-gram mouse. The toxicity of the penicillin sodium produced by manufacturers B to I, inclusive, falls between 5,000 and 21,000 units. In studying a number of lots of penicillin from different manufacturers over a period constituting the first three months of commercial production, it has been apparent that in some cases at least there has been a general decrease in the toxicity of the products produced. As an example, manufacturer E, who produced penicillin which was toxic at levels of from 7,000 to 8,000 units per 20-gram mouse in early production is now producing penicillin which is toxic for mice at from 14,000 to 16,000 units per 20-gram mouse. There has been a corresponding change in the potency. In early production the potency was from 200 to 300 units per milligram, while recent lots range from 700 to 1,000 units per milligram. Similarly, eleven lots of penicillin produced by manufacturer H, which assayed 200 to 500 units per milligram, were found to be toxic at 15,000 to 18,000 units per 20-gram mouse, while a more recent group of five lots assaying 134 to 160 units per milligram proved to be toxic in doses of 8,500 to 9,500 units per 20-gram mouse. This manufacturer recently reduced the purity of his preparation by changing his extraction method in order to increase his yield of penicillin.

In studies of six different salts of penicillin it has been shown⁹ that the toxicity is due mainly to the cation used in the preparation of these salts rather

than to the penicillin itself. Some evidence of this nature will be given later in this report. In the case of penicillin sodium the cation does not appear to be responsible, particularly with those products found toxic at low unitage. (See Table II.) In a previous study¹⁰ it was demonstrated that there are substances elaborated in the production of penicillin by the autolysis of the penicillium mycelia which are both pyrogenic and toxic for mice. It would appear, therefore, that the toxicity at lower unit levels of some manufacturers' products is associated with their methods of extraction of penicillin from the crude metabolic solution. It is of interest, however, that with manufacturer J's product, which was found to have an L_D-50 in mice (intravenous injection) of 28,000 units, the cation is responsible for the toxicity. This manufacturer's product had an average potency of 408 units per milligram and thus the L_D-50 in milligrams of penicillin sodium was 68.6. Since chemical assay showed the presence of 11.7 per cent Na^+ , there were 8.0 mg. Na^+ present at the L_D-50 . The L_D-50 of Na^+ of sodium acetate was found to be 7.8 milligrams. It is apparent, therefore, that the toxicity in this relatively atoxic product is primarily due to the cation.

COMPARISON OF THE ACUTE TOXICITY OF A CALCIUM AND A SODIUM SALT OF PENICILLIN

Through the courtesy of manufacturer I it has been possible to study the acute toxicity of a calcium and a sodium salt of penicillin, both prepared commercially from a single master lot of material. The potency of the calcium salt was found to be 310 units per milligram with a moisture content of 2.17 per cent, while that of the sodium salt was 332 units per milligram, with a moisture content of 2.0 per cent. Chemical assay of these salts showed the presence of 8.16 per cent Ca^{++} and 9.97 per cent Na^+ . To determine the acute toxicity of these two salts at least three mice were injected intravenously in the tail veins with increasing amounts of each salt until a level was reached at which the product was found to be no longer innocuous. The calcium salt was lethal for 20-gram mice in doses of 3,500 units, while a dose of 21,000 units of the sodium salt was required to bring about the same result. From a clinical standpoint this marked difference in toxicity of the sodium and calcium salts may have little significance since on a weight basis 3,500 units of the calcium salt would be equivalent to a single injection of over ten million units in a 60-kilogram man.

On the basis of potency it is obvious that the greater toxicity of the calcium salt cannot be associated with penicillin as such. During the preparation of the salts they had equal opportunity of picking up, from the master lot, substances toxic for mice. To what degree this occurred should be dependent upon their individual combining power with such substances. It would appear, however, that the demonstrated greater toxicity of the calcium salt is not the result of its selective combining power with toxic organic material in the master lot of penicillin since its purity (310 units per milligram) is not much less than the purity of the sodium salt (323 units per milligram).

In attempting to find the cause of the greater toxicity of the penicillin calcium, an L_D-50 was determined in mice for both the penicillin calcium and for calcium acetate. The L_D-50 for the calcium salt of penicillin was found to

be 3,700 units per 20-gram mouse. Since chemical assay showed the penicillin to contain 8.16 per cent Ca^{++} and its potency was found to be 310 units per milligram, the LD_{50} in terms of Ca^{++} is computed to be 0.97 milligrams. The LD_{50} of Ca^{++} of calcium acetate was found to be 1.03 mg. per 20-gram mouse. Therefore, the toxicity of the penicillin calcium is primarily due to the cation used in its preparation, and little, if any, toxicity can be ascribed to organic contaminants or to penicillin itself. Similar tests were made with penicillin sodium and with sodium acetate. In this case the sodium salt had a lethal dose of 21,000 units, a potency as noted above of 323 units per milligram, and it contained 9.97 per cent Na^+ . The number of milligrams Na^+ at the lethal dose was therefore 6.31. Since the lethal dose of Na^+ as sodium acetate was found to be 6.7 mg., it appears that the cation was largely responsible for the toxicity of this sample of penicillin sodium.

TABLE II

THE TOXICITY OF THREE SALTS OF PENICILLIN VS. THE TOXICITY OF THE CORRESPONDING ACETATES

MICE	UNITS PER 20 GM.	MG. PER 20 GM.	MORTALITY RATIO*	MICE	MG. PER 20 GM.	EQUIV. UNITS PENICILLIN PER 20 GM.	MORTALITY RATIO*
<i>Penicillin Sodium</i>				<i>Sodium Acetate</i>			
6	3,500	2.97	0/6	6	6.30	7,500	0/6
6	4,000	3.36	4/6	6	6.70	7,900	2/6
6	4,500	3.78	4/6	6	7.60	9,000	3/6
<i>Penicillin Magnesium</i>				<i>Magnesium Acetate</i>			
6	600	0.276	0/6	9	0.267	580	0/9
6	700	0.322	2/6	7	0.312	670	2/7
6	800	0.367	5/6	6	0.367	798	3/6
<i>Penicillin Ammonium</i>				<i>Ammonium Acetate</i>			
6	4,500	1.23	0/6	6	1.50	5,500	0/6
3	4,700	1.32	2/3	6	1.80	6,600	2/6
6	5,500	1.50	2/6	6	2.10	7,700	4/6
6	6,000	1.64	6/6				

*48-hour observations. Mortality ratio: Ratio of number of mice dying to total number injected.

COMPARISON OF THE ACUTE TOXICITIES OF THE SODIUM, MAGNESIUM, AND AMMONIUM SALTS OF PENICILLIN WITH SODIUM, MAGNESIUM, AND AMMONIUM ACETATES

In view of the results obtained above with a relatively atoxic lot of penicillin sodium, a comparison was made of a more toxic lot of penicillin sodium with sodium acetate. Since both penicillin magnesium and penicillin ammonium have been recommended for clinical trial, similar comparisons were made also with these two preparations. All three penicillin samples tested were commercial preparations. The tests were carried out in a manner similar to that recorded above and the results are given in Table II, where it will be noted that with the sample of penicillin sodium tested, 4,000 units caused death in mice. Since this product contained 84 mg. of Na^+ per 100,000 units, there were 3.36 mg. of Na^+ at the 4,000-unit dose. When comparison is made with sodium acetate it will be noted that the lethal dose of Na^+ of sodium acetate is 6.7 milligrams. Therefore, the toxicity of this sample of penicillin sodium is not due to any great extent to the cation, but rather to either penicillin itself or, more

likely, to organic contaminants resulting from the process of extraction, all of which can be removed by proper extraction methods. The results obtained with this sample of penicillin sodium are in marked contrast to those obtained on the sample of penicillin sodium referred to above which had a lethal dose of 21,000 units. It is of interest that the penicillin sodium referred to in Table II produces marked local reactions in the doses used clinically.

The penicillin magnesium preparation is extremely toxic in comparison to penicillin sodium or penicillin calcium. In the routine safety test, which consists of the injection of five 20-gram mice with 2,000 units each, all animals died within a few seconds, and it was not until the product had been diluted approximately 1:3 (to 600 units) that the preparation became innocuous. It should be pointed out, however, that the penicillin magnesium was a low-potency material (70 units per milligram). The L_{D-50} of this sample of penicillin magnesium was found to be 710 units per 20-gram mouse. Since the potency of this product was 70 units per milligram and it contained 3.22 per cent Mg^{++} , the number of milligrams of Mg^{++} at the L_{D-50} is therefore 0.325 milligrams. The L_{D-50} dose of Mg^{++} of magnesium acetate was found to be 0.38 milligrams. In the case of this preparation of penicillin magnesium, the toxicity of the product is primarily associated with the cation. Another sample of penicillin magnesium, prepared in these laboratories and having a potency of 1,028 units per milligram, was found to have an L_{D-50} for mice of 7,600 units. However, again with this high potency preparation, the cation appears to be responsible for the toxicity. Since the L_{D-50} was 7,600 units and the potency of the material 1,028 units per milligram, the L_{D-50} in milligrams of penicillin is 7.3 milligrams. Chemical assay showed the presence of 4.23 per cent of the cation. Therefore, at the L_{D-50} there were present 0.31 mg. of Mg^{++} , which corresponds quite closely to the milligrams of Mg^{++} present (0.38) at the L_{D-50} of magnesium acetate. The results obtained with both high and low potency preparations of penicillin magnesium tend to question the clinical use of such material particularly for intrathecal injections.

The lethal dose of penicillin ammonium is approximately 4,700 units (Table II). At this lethal dose there were found present by chemical analysis 1.32 mg. of NH_4^+ . By comparison, 1.8 mg. NH_4^+ were found to be present at the lethal dose of ammonium acetate. In the case of penicillin ammonium, the cation, although not primarily responsible for the toxicity of this preparation, probably contributed substantially to the toxic effect. The commercial preparation of penicillin ammonium was relatively insoluble in water, except in very low concentrations. When this preparation was dissolved in water at concentrations of 4,000 units per cubic centimeter a precipitate resulted which could not be dissolved even with the application of heat. The precipitate, however, contained little or no potency, since assay of the supernatant fluid indicated that all of the penicillin was in solution. In performing the tests given in Table II the penicillin ammonium was dissolved in water, the solution centrifuged, and the precipitate discarded before injection of the mice. However, the penicillin solution, on standing for a relatively short time, reprecipitated and it was not possible at any time during the study completely to clarify it. It is quite possible that this precipitate contributed to the toxicity of this

penicillin preparation. In any case it would appear unlikely that commercial penicillin ammonium in its present state of purity is satisfactory for clinical use in man.

SUMMARY AND CONCLUSIONS

A study of over 300 lots of penicillin sodium produced by fourteen manufacturers showed all to pass the mouse safety test.

There is a wide variation in the acute toxicity of different manufacturers' products. Some products are lethal for mice in concentrations of 3,500 to 5,000 units while one manufacturer is producing penicillin sodium lethal only at doses of 25,000 to 32,000 units.

A comparison of commercial samples of penicillin calcium and penicillin sodium made from a single master lot showed the calcium salt to be by far the more toxic preparation. The greater toxicity of the calcium over the sodium salt of penicillin should not discourage its clinical use in man. The toxicity of the penicillin calcium is primarily due to the cation.

The toxicity of high potency samples of penicillin sodium and high or low potency samples of penicillin magnesium is primarily due to the cation used in producing these preparations. Similarly, the cation contributes substantially to the toxicity of penicillin ammonium.

REFERENCES

1. Chain, E., Florey, H. W., Gardner, A. D., Heatley, N. G., Jennings, M. A., Orr-Ewing, J., and Sanders, A. G.: Penicillin as a Chemotherapeutic Agent, *Lancet* 2: 226, 1940.
2. Abraham, E. P., Chain, E., Fletcher, C. M., Gardner, A. D., Heatley, N. G., Jennings, M. A., and Florey, H. W.: Further Observations on Penicillin, *Lancet* 2: 177, 1941.
3. Hobby, G. L., Meyer, K., and Chaffee, E.: Chemotherapeutic Activity of Penicillin, *Proc. Soc. Exper. Biol. & Med.* 50: 285, 1942.
4. Herrell, W. E., and Heilman, D.: Tissue Culture Studies on Cytotoxicity of Bactericidal Agents, *Am. J. M. Sc.* 205: 157, 1943.
5. Herrell, W. E., Cook, E. N., and Thompson, L.: Use of Penicillin in Sulfonamide Resistant Gonorrheal Infections, *J. A. M. A.* 122: 289, 1943.
6. Blake, F. G., and Craige, B., Jr.: Penicillin in Suppurative Disease of the Lungs, *Yale J. Biol. & Med.* 15: 507, 1943.
7. Rammelkamp, C. H., and Keefer, C. S.: The Adsorption, Excretion and Toxicity of Penicillin Administered by Intrathecal Injection, *Am. J. M. Sc.* 205: 342, 1943.
8. Lyons, C.: Penicillin Therapy of Surgical Infections in the U. S. Army, *J. A. M. A.* 123: 1007, 1943.
9. Welch, H., Grove, D. C., Davis, R. P., and Hunter, A. C.: The Relative Toxicity of Six Salts of Penicillin, *Proc. Soc. Exper. Biol. & Med.* 55: 246, 1944.
10. Welch, H., Price, C. W., Chandler, V. L., and Hunter, A. C.: The Thermostability of Pyrogens and Their Removal From Penicillin, Restricted Circulation by O.S.R.D., Feb. 24, 1944.

ACTINOMYCOSIS

REPORT OF A CASE WITH MILIARY CHEST LESIONS

CAPTAIN ALBERT M. HARRIS, M. C., A. U. S., AND
MAJOR JOSEPH B. PRIESTLEY, M. C., A. U. S.

BOLLINGER, in 1877, first described the disease of cattle known as "lumpy jaw." He attributed the cause to *Actinomyces bovis*. In 1878 Esrael reported a case in man. The following year Ponfick showed the essential similarity of the organisms causing these conditions in man and cattle.

Actinomycosis enjoys a wide distribution, even though infrequent in occurrence. According to Cope,⁴ about 65 deaths result yearly from this disease in Great Britain. Auster¹ found the condition more prevalent in grain-producing areas of the United States. Davis,⁵ however, reports an even distribution of actinomycosis between rural and urban populations.

The actinomyces constitute a very commonly encountered group of organisms. Zinsser and Bayne-Jones¹¹ found it in soils and occasionally in normal mouths. Microscopically, the organism has a branching filamentous structure with a peculiar predisposition to form into clumps or colonies. The central portion of these clumps appears as a mass of debris with radiating filaments and club forms around the periphery. This structure gives rise to the term "sulfur granules." Generally these sulfur granules can be seen grossly and are considered diagnostic. Actinomycosis really connotes a group of diseases, just as the actinomyces belong to a group of organisms rather than a single pure strain. Some of these organisms have aerobic qualities, while others are anaerobic. Most of them take the Gram stain. A few are acid-fast. The fact that several types of organisms occur has led to considerable controversy. Likewise, numerous names have been given to different forms of the disease caused by these various strains. The following quotation from Foulerton⁷ indicates an early appreciation of the confusion: "Whilst in this country and the United States the term streptotrichosis has been adopted generally, continental pathologists as a rule still favour the older designation for these infections, actinomycosis. And, writers on general medicine have not always appreciated the fact that these two terms are absolutely synonymous; so that one sometimes finds even in recent books reference made to the streptotrichoses as if they were in a class of disease in some sort different from the actinomycosis."

Recently the controversy regarding the classification and nomenclature of the *Actinomyces* has been greatly clarified by the work of Waksman and Henri¹⁰. According to their classification, the common type, as seen in lumpy jaw, has anaerobic characteristics and receives the name of actinomycosis. The aerobic form with the tendency to reproduce by fragmentation of the mycelium is designated "Nocardia." They also mention its frequent acid-fast features.

The diagnosis rests on finding the organism in the lesions. Usually one sees the typical "sulfur granules." When difficulty in finding sulfur granules arises, however, stains may be employed to demonstrate the individual organisms. Pathologically, the lesions produced are characterized by a large amount of fibrosis, some necrosis, and the presence of a purulent exudate containing polymorphonuclear leucocytes. The picture somewhat resembles tuberculosis and other granulomatous lesions, except for the more acute type of exudate. Sometimes giant cells appear.

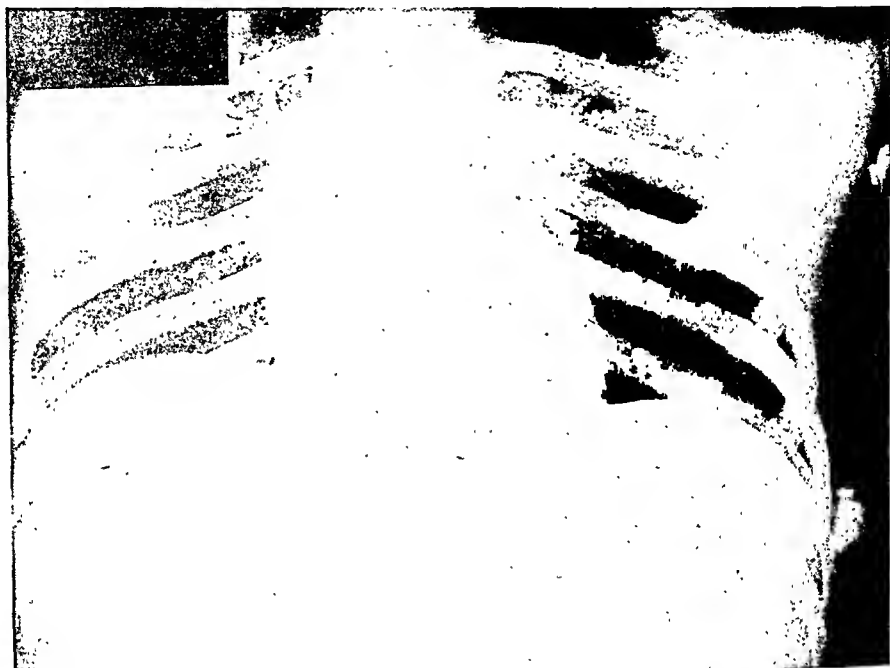


Fig. 1.—Photograph of roentgenogram of the chest and upper abdomen, showing miliary actinomycosis.

Cecil³ groups cases of actinomycosis according to their clinical manifestations in the following manner: (a) The most common type involves the head and neck. It comprises 50 per cent of the reported cases. The mouth serves as the source of infection. (b) The next most common type comprising 20 per cent to 30 per cent of all cases occurs in the abdomen. This type most frequently follows an appendectomy or other similar procedure. Characteristically, numerous sinuses develop. (c) The third, or pulmonary, type comprises approximately 15 per cent of the total. In addition to these three more common types, other rare forms involving the skin or mucous membranes and the generalized form occur. Most writers on the subject mention the possibility of generalized spread.

In 1934 Biggart² reported the case of a woman who developed a lesion on the thigh. The infection spread rapidly and death occurred within three months. The autopsy findings included generalized actinomycosis lesions of the lungs, liver, spleen, and kidneys. The case herewith presented has similar features.

The disease commonly runs a chronic course lasting from months to years. There have been a large number of complete recoveries reported. In general, however, the prognosis is not too favorable. According to Davis,⁵ the cervico-facial type offers the best prognosis. Wangensteen⁹ emphasizes the poor prognosis of actinomycosis involving the chest or abdomen.

In the treatment the single most important factor is good surgical care. Wangensteen⁹ emphasizes the importance of this. According to him, as the organism is an anaerobic one, the removal of dead tissue and debris is necessary in order to do away with media for the growth of the organism and to aerate the tissues. Numerous drugs have been used. Dobson⁶ found sulfanilamide helpful in some cases. The action of the drug in actinomycosis appears to be on the secondary invaders rather than the primary infection. Joyce⁸ recommends the use of thymol in conjunction with radical surgery. A number of authors, including Dobson, recommended radiation with the thought that the resulting fibrosis tends to wall off the infection. The iodides have been used for some years. Best modern opinion regarding treatment seems to be the use of radical surgery in connection with thymol and radiation.

CASE REPORT

The soldier, a Negro male aged 23 years, entered the hospital June 9, 1943, complaining of a painful swelling in the left axilla of eight days' duration. He recalled no injury. Previous to admission he received several days' treatment with hot packs and sulfathiazole; the condition grew worse.

The salient features of the physical examination on admission consisted of several firm, tender, nonfluctuating, enlarged axillary glands. One group felt conglomerated. The temperature registered 102°, the pulse 88, and respiration 20 per minute. Examination of the blood showed 19,000 white blood cells with a secondary anemia.

Four days after admission, incision of the left axilla produced 30 c.c. of thick, white pus. No sulfur granules were visible grossly and the culture grew no organisms. Early treatment, consisting of incision and drainage with chemotherapy, resulted in no improvement. The high temperature continued. The blood culture was reported negative. Ten days later the axilla was again incised and a second incision on the anterior portion of the chest was made. This drained several pockets and infected the tracts. Through-and-through drainage was established, and a piece of tissue was removed for biopsy. Upon re-examination of the pus and from the biopsy, the diagnosis of actinomycosis was established. Patient then received iodides both orally and by vein, thymol, transfusions, and plasma. No improvement was observed, however. The temperature remained very high, seldom below 101°. The white blood cells numbered 35,000. The number of immature forms increased as the condition progressed. Four weeks after admission an enlargement resembling a left inguinal adenitis appeared. This immediately suggested a metastatic abscess. Upon incision a few days later, pus disgorged. In spite of excellent nursing care the patient developed two decubitus ulcers on the back. Roentgen therapy produced no beneficial results. One month after admission, the presence of pulmonary findings was noted. Chest films at this time revealed multiple deposits distributed evenly through the parenchyma of both lungs, although a film eleven days previously was reported negative. Frequent nosebleeds became troublesome. Death occurred seven weeks after admission, following several irrational days.

Laboratory diagnosis was made on the basis of smears and tissue sections. The smears showed the frequent presence of an acid-fast organism. This organism was a long, slender rod with a fairly marked tendency to produce poorly developed buds. Except for the budding tendency, the large number of organisms present, and the predominance of cells with a segmented nucleus, the picture resembled that commonly seen with the tubercle bacillus. Sections of the actinomycotic tissue showed a ground work of relatively avascular gran-

ulation tissue. Fibroblasts, reticulocytes, and endothelial cells predominated. Scattered through the tissue appeared numerous small areas of necrosis surrounded by many polymorphonuclear neutrophils. No organisms took the ordinary hematoxylin-eosin stains. Acid-fast stains showed the same organisms seen in the smears. Mallory's stain for actinomycosis was not helpful, except for one slide which had a small, but typical colony of so-called "sulfur granules."

A guinea pig inoculated with material from the sinus tract died the same day as the patient. It showed lesions quite similar to those seen in the patient. The organisms demonstrated in the guinea pig tissues were identical to the organisms seen in the human tissue. No growth was obtained on Sabouraud's or other media commonly used for fungi.

AUTOPSY FINDINGS

External Examination.—This is the body of a fairly well-developed, thin, young adult Negro male estimated to be 5'7" tall and weighing approximately 130 pounds. There is an ulcerated area in the left inguinal region with an opening of a sinus tract. There is a decubitus ulceration in the left costovertebral angle and also over the sacrum. There is a 9 cm. recent surgical incision just lateral to the border of the pectoralis muscle on the left. This leads into a sinus tract in the soft tissues over the chest. There is a 2 cm. incision in the belly of the muscle to provide through-and-through drainage.

Peritoneum.—The liver border is 4 cm. below the right costal margin. The spleen is at the left costal margin. The peritoneal cavity contains about 50 c.c. of blood-tinged serous fluid. There are no adhesions in the peritoneal cavity.

Pleural Spaces.—On removing the sternum the thoracic duct is seen to be swollen and filled with a mass of inflammatory tissue. Pleural spaces contain no excess free fluid. There are no adhesions in either pleural space.

Heart.—Essentially normal.

Lungs.—The right lung weighs 915 grams. There is some atelectasis posteriorly. The hilar lymph nodes are enlarged. Bronchi are filled with blood-tinged frothy material. The cut surface presents a congested appearance with considerable edema and multiple miliary lesions. There is considerable consolidation of the tissue.

The left lung weighs 820 grams. The atelectasis is least marked. The cut surface is essentially similar to the right. In some areas there is beginning cavitation. The miliary lesions are uniform in size, the largest measures approximately 3 mm. in diameter.

Liver.—Weight of the liver is 2,310 grams. The capsular surface is studded with miliary lesions. The cut surface is also completely taken up with these lesions. All of them are small and the largest measures approximately 1 mm. in diameter.

Spleen.—Weight of the spleen is 480 grams. The capsular and cut surfaces are studded with many miliary lesions, the largest measures approximately 4 mm.

Pancreas.—Normal.

Kidneys.—The right kidney weighs 195 grams. The left kidney weighs 240 grams. There are fairly numerous miliary lesions in both kidneys. The largest measures about 3 mm. The lesions are similar to those seen in other organs except there are not so many of them in the kidney as in other tissues.

Adrenals.—The medullary portion is hard to differentiate from the cortex.

Miscellaneous.—There is some enlargement of the mediastinal and mesenteric lymph nodes. There is a mass of enlarged lymph nodes in the region of the celiac axis. These lymph nodes on cut section present a matted necrotic appearance. Several areas in the nodes contain purulent material. Dissection of the left axilla shows the sinus to be multilocular and to extend beneath the scapula and into the subclavicular region. It does not, however, penetrate the pleura.

MICROSCOPIC SECTIONS

Heart.—Normal.

Lungs.—Scattered through the lung tissue, rather uniformly, are small areas of necrosis. These areas have some mononuclear reaction surrounding them; in general, however, the cellular response is minimal. An occasional multinucleated giant cell of the Langhans type is seen. Miliary lesions are scattered throughout all the lung sections.

Liver.—Scattered through the liver sections are miliary lesions which are similar to those described in the lung. The cellular response is again minimal.

Spleen.—The spleen contains a large number of miliary lesions similar to those seen in the lung. Many of the lesions in the spleen are larger than those seen in the lung or liver. In other respects they are similar.

Pancreas.—There are, in isolated areas, occasional miliary lesions similar to those seen in other organs.

Kidney.—Scattered through the tissue there are fairly numerous miliary lesions similar to those seen in other organs. It is noted that these lesions are frequently in the areas of the arcuate arteries or in the pyramids.

Adrenals.—Adrenals are normal, except for the presence of fairly numerous miliary lesions similar to those seen in other organs.

Lymph Nodes.—Sections through the lymph node show a much more passive necrosis than that seen in other areas. The follicular structure is almost entirely replaced. Edema is a fairly prominent feature. Again the paucity of a cellular response is noted.

Miscellaneous.—Acid-fast stains were done on many of these tissues. These sections show the presence of an acid-fast organism in all of the areas where miliary lesions are encountered. These organisms are for the most part long slender nodes with a tendency to appear in small groups or clumps and a marked tendency to produce branching forms. The large number of organisms encountered is an interesting feature. Another characteristic is the tendency for the organisms to be distributed widely. Mallory's stain for actinomycosis was not particularly helpful.

CONCLUSIONS

1. A case of generalized actinomycosis showing interesting miliary lung lesions is reported, with autopsy findings.

2. The first clinical manifestation appeared in the axillary glands, suggesting the infection entered an abrasion of the hand or arm.

3. The final pathologic picture resembled in many respects the end result in miliary tuberculosis. The finding of inflammatory tissue in the thoracic duct suggests this origin for the hematogenous spread.

4. All modern methods of treatment were employed, including adequate surgery, chemotherapy, roentgen therapy, thymol, iodides, and supportive treatment. None of these appeared appreciably to affect the progress of the disease.

REFERENCES

1. Auster, Lionel S.: Actinomycosis, *Am. J. Clin. Path.* 10: 652, 688, 1940.
2. Biggart, J. H.: Actinomycosis Gruminis, *Bull. Johns Hopkins Hosp.* 54: 165, 1934.
3. Cecil, Russell L.: *Textbook of Medicine*, 5th Ed., Philadelphia, 1942, W. B. Saunders.
4. Cope, Zachary: Actinomycosis, New York, Oxford University Press, 1938.
5. Davis, M. I. J.: Analysis of 46 Cases of Actinomycosis With Special Reference to Its Etiology, *Am. J. Surg.* 52: 447-454, 1941.
6. Dobson, L., Holman, E., and Cutting, W. C.: Sulfanilamide in the Therapy of Actinomycosis, *J. A. M. A.* 116: 272-275, 1941.
7. Foulerton, A. G. R.: Some Observations on a Series of 78 Cases of Streptothrix Infection, *Lancet* 184: 381, 1913.
8. Joyce, T. M.: Thymol Therapy in Actinomycosis, *Ann. Surg.* 108: 910-917, 1938.
9. Wangeunsteen, O. H.: The Role of Surgery in the Treatment of Actinomycosis, *Tr. Am. S. A.* 54: 279-297, 1936.
10. Waksman, Selman A., and Henrici, Arthur T.: The Nomenclature and Classification of the Actinomycetes, *J. Bact.* 46: 337-341, 1943.
11. Zinsser, H.: *Textbook of Bacteriology*, 8th Ed., New York, 1943.

LOCAL EOSINOPHILIA IN MALIGNANT NEOPLASMS

A. J. GILL, M.D., DALLAS, TEXAS*

IT IS the purpose of this paper to review briefly the question of local eosinophilia in cases of malignant tumor, especially as observed in carcinoma of the cervix uteri.

Eosinophile infiltration has been observed in many instances in the stromal tissues of a variety of neoplasms. I have noted that the phenomenon is most striking and most frequent in cases of "epidermoid carcinoma" of the cervix. In the typical case, which shows this special reaction, the neoplasm itself is not remarkable in appearance and there is no very impressive histological peculiarity which sets such tumors apart, except for the marked local eosinophilia. I am not aware, either, of any unusual features of clinical appearance or history in these cases prior to biopsy.

The inflammatory reaction in the stroma of carcinoma of the cervix is somewhat variable, although, in most instances there is a rather marked infiltration of lymphocytes, plasma cells, and moderate numbers of large mononuclear cells. Polymorphonuclear neutrophils occur with somewhat less frequency. Eosinophiles are present in the vast majority of cases, but usually are few in number, and widely scattered. They are probably not much more numerous than might be expected in many chronic inflammatory processes. In a small percentage of cases these cells appear to be absent. In another small group of carcinomas of the cervix, there is an infiltration of eosinophiles which is definitely greater than that seen in the great majority and which, in a few cases, exceeds the number of all other inflammatory cells present.

The characteristic lesion of the type under consideration reveals a moderately undifferentiated epidermoid carcinoma, in the stroma of which there is a dense infiltration of typical eosinophiles. Occasionally the tumor stroma is entirely obscured by the large number of these cells which lie closely packed together.

From January 1, 1938, to August 4, 1943, there have been approximately 361 biopsy fragments of carcinoma of the cervix received in this laboratory, of which 317, from 309 patients, were available for study in the preparation of this paper. Only eight of the 317 cases studied were adenocarcinomas and none of the eight showed the change in question. Each slide has been examined and an estimate made as to the frequency with which eosinophiles appear among the inflammatory cells in the stroma. As noted in a previous paragraph, most of the cases reveal small numbers of eosinophiles. An effort was made to pick out only those in which eosinophilia was definitely greater than could be seen in the majority of the slides studied. It was decided to attempt further division of the cases, which showed eosinophilia greater than usual, into four grades of eo-

From the University of Tennessee, Department of Pathology, Memphis, Tennessee.
Received for publication, March 14, 1944.

*At present at Southwestern Medical Foundation School of Medicine, Dallas, Texas.

sinophile density, designated one plus to four plus. This division was based upon an estimate of numbers of cell types in all parts of the tumor stroma. It should be mentioned at this point that the distribution of inflammatory cells is often variable and there are sometimes rather marked differences in the type of exudate from one part of a section to another part of the same section.

I have chosen to base the one plus to four plus degrees of eosinophilic infiltration upon the following estimates of numbers—i.e., one plus equals any increase in eosinophiles above that seen in the majority of cases, to about 25 per cent of the total inflammatory cells present; two plus equals an estimated 25 to 50 per cent eosinophilia; three plus equals 50 to 75 per cent eosinophilia; four plus equals 75 per cent or greater infiltration of eosinophiles. I wish to emphasize that these figures do not represent actual counts of cells, but are estimates based upon a survey of all parts of a single slide in each case.

Of the 317 biopsies studied, a total of 26 slides, from 24 patients, revealed a local eosinophilia which definitely exceeded that commonly seen in the other 291 biopsies. The subdivision into groups revealed the following: one plus, 17 cases; two plus, 2 cases; three plus, 3 cases; four plus, 4 cases. Thus it can be seen that 7.7 per cent of the cases in this series show what I believe to be a significant local eosinophilia; and in 7 (2.2 per cent) of these, it appears that eosinophiles make up more than half of all inflammatory cells present. There is almost invariably a heavy inflammatory cell infiltration in those cases of marked local eosinophilia, and there were, therefore, very large numbers of these cells present.

From January 1, 1938, to September 21, 1939, there were 104 biopsies of carcinoma of the cervix studied from a total of 97 patients. Two of this group were adenocarcinomas. Eight biopsies revealed significant eosinophilia in a total of six patients. Three biopsies from one patient over a period of about six weeks, each revealed a marked eosinophilic reaction.

Complete follow-up information on cases from January 1, 1938, to September 21, 1939, was available on 53 of the patients of the original group of 97. Only one of this group of 53 is alive at present. This case failed to show the inflammatory reaction under consideration. Final follow-up data were available on all 6 of the patients who showed noteworthy eosinophilia in the biopsy tissue. Analysis of the 53 cases reveals that the average duration of life after biopsy of the 6 notable cases was approximately seventeen months, whereas the remaining 47 lived an average of approximately fourteen months. This is perhaps suggestive but not considered a definitely significant difference in such a small series. There appears to be no important difference in longevity between various degrees of local eosinophilia in this series.

It is obvious that there are many opportunities for error and misinterpretation of findings in a group of this type. There is, of course, a large personal factor in the judgment of what does and does not constitute a degree of local eosinophilia in the one plus group of cases, which is definitely greater than that observed in the majority of slides. Another possibility for error lies in the smallness of the series of patients on whom up-to-date data could be obtained. It is possible and even probable that those patients who cannot be traced are more likely to be alive and are lost to sight because of change of address, name.

etc. It is probable, then, that some of the ninety-seven patients on whom follow-up data was not available, are still living although all but one of the fifty-three patients traced successfully to date are dead. In determining longevity, based upon length of life, from biopsy to death there is undoubtedly considerable error because of the wide differences that exist in various patients as regards their response to early symptoms of disease and the time which was allowed to elapse between onset of symptoms and appearance of the patient in a clinic for biopsy.

It appears that the type and extent of the inflammatory reaction seen in connection with carcinoma of the cervix, probably does not depend upon any obvious features of invasion, character of tumor, or degree of superficial necrosis except where the biopsy fragments include necrotic debris and exudate from surface ulceration. In the superficial tissues, of course, where extensive ulceration and secondary infection are marked, there is usually an exudate, the cellular portion of which is composed chiefly of polymorphonuclear leucocytes. This is usually quite different from the type of inflammation which occurs deep in the stroma and which may represent resistance to tumor invasion. In a very few instances there is little or no inflammation in the stroma of the tumor. Most of this latter group tend to be rather well-differentiated tumors and often reveal an extremely dense, relatively acellular type of stroma.

It has been felt by many observers that inflammation in neoplastic processes might represent resistance of the host. Ewing² notes that inflammation often meets the invasion of tumor tissue and that this is a significant defensive process.

It is interesting to speculate as to the causes of appearance of inflammatory cells in general and eosinophiles in particular as a response to tumor invasion. Dead and dying cells and many bacteria are chemotactic for inflammatory cells and it is not unlikely that degenerations of tumor tissue alone might account for exudate in and about the tumor in many cases. As soon as ulceration and secondary infection occur there is ample explanation for infiltration of inflammatory cells of various types. As regards the attraction of eosinophiles, Ingraham and Wartman⁴ observed that eosinophiles are strongly attracted by certain bacteria and other substances in a manner comparable to the response of neutrophils to the same chemotactic substances. They suggest that it is possible that eosinophiles may be attracted to a site of inflammation in the same manner as polymorphonuclear neutrophils.

Apropos of tissue breakdown and foreign protein reaction, it has been shown by Biggart¹ that foreign protein injected in muscle results in a polymorphonuclear leucocyte response about the site within twenty-four hours, but at the end of about eight days the reaction is predominately eosinophilic. It appears, however, that in animals already showing a general eosinophilia, the injection of proteins causes a local reaction which may be predominately eosinophilic from the first. Sterile necrosis from turpentine injection gives similar results.

The question as to the origin of eosinophiles has come up from time to time in the past and there has been some difference of opinion as to their histogenesis. It is believed by some that tissue eosinophiles may be developed from lymphocytes, plasma cells and perhaps other cell types in the tissues and that many

eosinophiles seen in various conditions are formed locally and do not necessarily arrive from the blood. Biggart¹ feels that there is no distinguishing feature between eosinophiles found in tissues and those of blood. He concludes that the tissue eosinophile "is an emigrated eosinophile leucocyte" and that "local eosinophilia occurs at the expense of the circulating eosinophiles." He lists many conditions in which he has observed eosinophilia. His list includes among others, inflamed appendices; chronic gastric ulcer; epithelioma of hand, head, and tongue; carcinoma of the thyroid, and a variety of carcinomas of the gastrointestinal tract. He makes the interesting observation that every neoplasm of the gastrointestinal tract examined by him showed a local eosinophilia, regardless of the presence or absence of secondary inflammatory changes.

The suggestion is made by Biggart that aseptic necrosis of an animal's own tissues will set free chemotactic factors, probably protein in nature, for eosinophiles; and that eosinophilia in tumors, whose protein metabolism is especially active, and in which there is constant aseptic destruction of normal and malignant tissue cells, probably is of this nature.

A number of reports have appeared which suggest some immediate value to be derived from the observation of unusual eosinophilia in connection with neoplastic diseases, quite apart from the more academic questions as to the why and wherefore of the phenomenon. Goforth and Snook² regard the accumulation of many eosinophiles locally as evidence of body resistance to neoplasia. They report a case of carcinoma of the cervix with marked local eosinophilia in which the patient exhibited prolonged resistance to the progress of the tumor process, and they state that in instances where local eosinophilia has been a feature, the patient has shown greater resistance to the process, has responded better to treatment and lived longer than when eosinophilia was absent. It is their opinion that the presence of the eosinophile is a good omen in carcinoma of the cervix.

Schoeh³ examined 417 cases of carcinoma of the cervix by biopsy and noted eosinophilia in 10 per cent. Three hundred fifty-nine of the patients in this series died in five years. Of this number 9 per cent showed eosinophilia. In the cases which remained cured for five years (58 cases) eosinophilia was noted in 40 per cent. The proportion of cures in the total number of cases (417) was 13.2 per cent; in the 40 cases with eosinophilia, cures were 45 per cent. All cases received radiotherapy. These are interesting observations although there appears to be some discrepancy in the figures.

Pavlovsky and Widakowich⁴ observe that eosinophiles seem to have a protective action against cancer invasion and that in malignant disease with marked eosinophilia, the course is more favorable.

The causes of the marked local infiltration of eosinophiles in some cases of malignant neoplasm are, as yet, obscure. None of the usual explanations for eosinophilia seem entirely adequate. It is possible that special conditions of necrosis with liberation of protein degeneration products, or unusual bacterial infection of ulcerated lesions, or perhaps a strong allergic susceptibility of an individual to either protein products of degenerated tissues or bacteria, may account for this phenomenon. It is unlikely, in my opinion, that there is any special tumor tissue which is specifically chemotactic for eosinophiles. It seems

much more reasonable to assume that the reaction is due to some change, probably degenerative, in the tumor or in the tissue of the host or in both. This may not be different from an allergic reaction of the host to a protein antigen.

SUMMARY

What is thought to be a significant degree of local eosinophilia was observed in 7.7 per cent of a series of 309 cases of epidermoid carcinoma of the cervix.

There is some reason to believe that the presence of eosinophiles in abundance in the stroma of malignant tumors is of good prognostic import (other factors being equal), and probably represents a better than usual resistance to the advance of the neoplasm.

It is hoped that future long-term observations with follow-up cases will confirm the value of this phenomenon as a favorable indication in prognosis.

REFERENCES

1. Biggart, J. H.: *Some Observations on the Eosinophile Cell*, *J. Path. & Bact.* 35: 599-816, 1932.
2. Ewing, James: *Neoplastic Diseases*, ed. 4, Philadelphia, 1940, W. B. Saunders Co., page 35.
3. Goforth, J. L., and Snoke, P. O.: *A Consideration of Body Resistance to Neoplasia*, *Am. J. M. Sc.* 175: 504-510, 1928.
4. Ingraham, E. S., and Wartman, W. B.: *Chemotropism of Human Eosinophilic Polymorphonuclear Leucocytes*, *Arch. Path.* 28: 318-322, 1939.
5. Pavlovsky, A. J., and Widakowich, V.: *Local Eosinophilia in Cancer; Are the Eosinophile Cells Oncolytic Elements?* *Semana med.* 1: 1265-1278, 1926.
6. Schoch, E. O.: *Local Eosinophilia in Carcinoma*, *Zentralbl. f. Gynäk.* 50: 2895-2900, 1926.

SUBACUTE BACTERIAL ENDOCARDITIS CONFINED TO A PULMONIC VALVE WITH MALFORMED LEAFLETS

R. J. ROGERS, M.D., HINES, ILL.

ENDOCARDITIS confined to the pulmonic valve is of infrequent occurrence, single cases having been reported by De Candole,¹ Lehman,² Leader,³ Grayzel,⁴ and Cockayne and Wilton.⁵ In all but one of these, Leader's Case, the endocarditis was of the acute variety in keeping with Allen's⁶ observation that there seems to be a generally greater tendency for the virulent rather than the relatively avirulent organisms to involve the right side of the heart. Libman⁷ reports that 26.8 per cent of cases of acute endocarditis involve the right side of the heart, and that only 1 per cent of the subacute cases affect that side. Denman⁸ and Thayer⁹ reporting separately on subacute endocarditis, place the incidence of cases in which the pulmonic valve alone is affected at 2 per cent and 1 per cent, respectively. Clawson and Bell¹⁰ found no cases confined to the pulmonic valve in their series of eighty cases.

Aside from its unusual location as a subacute lesion, the following case of endocarditis presents findings of more than passing interest, foremost of which is a pulmonic valve in which two of the leaflets are rudimentary. Another interesting feature is the fact that the right side of the heart showed no evidence of hypertrophy or dilatation, despite the marked pulmonary valve insufficiency caused by the defective leaflets and the superimposed inflammatory destruction.

CASE REPORT

The patient, F. B., was a white male, aged 47 years, a meat cutter by occupation.

The family history contained nothing of significance. The past history states that he had had tonsillitis and joint pains as a young man. He had always been weak, asthmatic, and troubled with dyspnea.

Present Illness.—About six months before admission to the hospital he developed a dry cough with occasional hemoptysis. One week before admission he became ill with chills, fever, malaise, aching in muscles and joints, pleuritic pains, nausea, vomiting, and loss of appetite. He had lost twenty pounds in the last six months. After hospitalization the patient had intermittent febrile episodes, the temperature ranging from 99° F. to 105° F. He complained of cough, and pain in the chest and abdomen.

Physical Examination.—The patient was pale, emaciated, ill appearing, with no cyanosis, dyspnea, or edema. There were dullness on percussion, harsh breath sounds, râles, and a pleural friction rub in the left lower chest. The peripheral vessels were not sclerosed, the pulse rate was 120; rhythm regular. The apex impulse was in the fifth interspace 8 cm. from the midsternal line. The pulmonic second sound was accentuated with reduplication. There was a three plus rather harsh systolic murmur over the pulmonic area. There was a soft high-pitched diastolic murmur along the left sternal border. The liver and spleen were not palpable.

Published with the permission of the Medical Director of the Veterans' Administration who assumes no responsibility for the opinions expressed or conclusions drawn by the author from the Department of Pathology, Veterans' Administration, Hines, Illinois.

Received for publication, March 9, 1944.

much more reasonable to assume that the reaction is due to some change, probably degenerative, in the tumor or in the tissue of the host or in both. This may not be different from an allergic reaction of the host to a protein antigen.

SUMMARY

What is thought to be a significant degree of local eosinophilia was observed in 7.7 per cent of a series of 309 cases of epidermoid carcinoma of the cervix.

There is some reason to believe that the presence of eosinophiles in abundance in the stroma of malignant tumors is of good prognostic import (other factors being equal), and probably represents a better than usual resistance to the advance of the neoplasm.

It is hoped that future long-term observations with follow-up cases will confirm the value of this phenomenon as a favorable indication in prognosis.

REFERENCES

1. Biggart, J. H.: Some Observations on the Eosinophile Cell, *J. Path. & Bact.* 35: 599-816, 1932.
2. Ewing, James: *Neoplastic Diseases*, ed. 4, Philadelphia, 1940, W. B. Saunders Co., page 35.
3. Goforth, J. L., and Snoke, P. O.: A Consideration of Body Resistance to Neoplasia, *Am. J. M. Sc.* 175: 504-510, 1928.
4. Ingraham, E. S., and Wartman, W. B.: Chemotropism of Human Eosinophilic Polymorphonuclear Leucocytes, *Arch. Path.* 28: 318-322, 1939.
5. Pavlovsky, A. J., and Widakowich, V.: Local Eosinophilia in Cancer; Are the Eosinophile Cells Oncolytic Elements? *Semana med.* 1: 1265-1278, 1926.
6. Schoch, E. O.: Local Eosinophilia in Carcinoma, *Zentralbl. f. Gynäk.* 50: 2895-2900, 1926.

Microscopic.—Serial sections taken from the left cusp, Fig. 2, showed almost complete absence of the leaflet, except for a very small budlike projection of fibrosa. This was rounded and smooth, covered by endothelium, and an intact elastic lumen could be demonstrated under the endothelium with Weigert's elastic tissue stain.

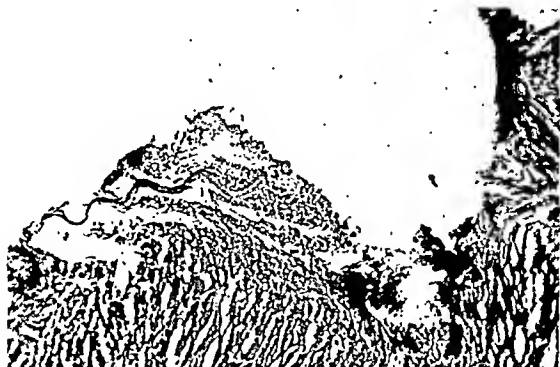


Fig. 2.—Microphotograph of section through the left pulmonary cusp. The subendothelial elastic lamina is clearly defined in the budlike leaflet. Weigert's elastic tissue stain $\times 300$.



Fig. 3.—Photomicrograph of section through the right pulmonary cusp. There are many neutrophils and phagocytes within the fibrosa with loss of structure. Part of a small vegetation with its group of polymorphonuclears is seen at the lower edge. Hematoxylin and eosin $\times 300$.

Sections taken from the right cusp, Fig. 3, showed a hyalinized vegetation attached to the lumen side of the leaflet, within which many polymorphonuclear neutrophils were to be seen. The valve spongiosum (not seen in the illustration) showed the presence of many newly

formed capillaries and young fibroblasts. No evidence of rheumatic involvement could be demonstrated in serial sections of the myocardium and leaflets. Bronchopneumonia and infected emboli could be demonstrated in the sections of lung.

DISCUSSION

Since no rheumatic stigmata were found either grossly, or in serial microsections of the valves and myocardium, it is quite obvious that the "locus minoris resistentiae" for the *Streptococcus viridans* was the defective pulmonary valve leaflets. To determine whether these defects were congenital or acquired requires careful analysis of the gross and microscopic findings. The fact that no other anomalies were present in the heart is of little significance. Abbott,¹¹ in her tabulation of one thousand cases of congenital heart disease, reported 43 cases of congenital bicuspid valves unaccompanied by other defects and 125 cases in which other anomalies were noted. We may conclude from Abbott's findings that the presence of other congenital anomalies makes it more likely that a given lesion is congenital, while their absence does not exclude the probability of a congenital etiology. De Vries¹² considered that all cases in which the pulmonary valve is affected (by endocarditis) are due to maldevelopment. Koletsky,¹³ referring to bicuspid pulmonary valves, states that they are all of congenital origin and that as far as is known, no acquired deformity of this type has ever been observed.

The microscopic findings strongly indicate, if they do not prove, that the defective leaflets are of congenital origin.

Fig. 2 is a photomicrograph of a section through the budlike prominence representing the rudimentary left leaflet. The fibrosa is covered by unbroken endothelium and the elastic laminae are intact. These findings, particularly the latter, are not what would be expected in inflammatory destruction followed by repair. It is true that the intact endothelium means little by itself, since regeneration of endothelium is a common accompaniment of intimal repair; however, it is hardly conceivable that extensive inflammatory destruction would leave an intact subendothelial elastic in the remaining portion of the leaflet, nor is it likely that repair would so closely simulate the normal relationship between fibrosa elastica and endothelium.

Assuming for the moment that the cusp remnants are congenital anomalies, we are offering the following hypothesis taken in part, from Simonds¹⁴ to explain their maldevelopment. In the developmental division of the common arterial trunk into the aorta and pulmonary artery, two pairs of swelling develop on the inner surface of the vessel. These four swellings are anlagen of the semilunar cusps; later the lateral pair become divided in their longitudinal axis when the common trunk forms the aorta and pulmonary artery; this results in two groups of three pads, one group in each vessel. The semilunar leaflets are formed when these pads are indented, or hollowed out from above. Incomplete hollowing or shallow indentation of the pads from above would produce defects in which the leaflet would be relatively shallow or deep, according to the point at which the process was arrested.

Definite proof that a given lesion of a heart valve is congenital is possible in some instances. For bicuspid aorta and pulmonary valves, particularly for the former, well-defined gross and microscopic criteria have been developed to differ-

entiate between congenital and acquired lesions. The work of Bishop and Trubek¹⁵ and later by Koletsky¹⁶ provides definite gross and microscopic differentiating points, the main one being the relationship in the raphe of the elastic media with the annulus fibrosa. In congenital cases the media is superficial to the connective tissue of the annulus fibrosa, which is the reverse of the normal relationship, in which the annulus is superficial to the elastica. Obviously, such criteria is of no help in determining the nature of the defective leaflets found in our case since we are not concerned here with a raphe; however, the microscopic findings, the intact subendothelial elastica, and the absence of signs of inflammation in the left cusp seem adequate to establish the defect as congenital, while that in the right cusp, with all the evidence here of inflammatory destruction, may be purely inflammatory or due to combined maldevelopment and inflammation.

The *a priori* effect of a pulmonary valve insufficiency of over 60 per cent, i.e.; almost total loss of two leaflets, would be hypertrophy, dilatation, or both, of the right auricle and ventricle. As noted in the case report and autopsy, such effects were not present. The electrocardiogram was normal, and the heart was of normal size and shape without evidence of hypertrophy or dilatation in any of its chambers. This apparent incongruity demands some explanation. Fig. 1 shows the defective pulmonic valve and the large polypoid vegetation on its stalk. It was noted at autopsy that the globular dependent portion of the vegetation occupied the space within the valve ring usually closed by the right and left leaflet. It seems probable that the vegetation had some compensatory effect on the insufficiency by inhibiting the back flow of blood into the right ventricle during diastole; in other words the mobile, rounded vegetation functioned somewhat as a ball valve, moving upward with the blood stream in systole and dropping back into the ring in diastole, thus serving to diminish the effects of the incompetent leaflets. Another point to consider is the probability that the valve was competent before the onset of the endocarditis. This seems likely since many congenital defects are silent clinically; e.g., in Koletsky's report of nine cases of congenital bicuspid pulmonary valves, he states that they were silent and were found to be of little, if any, clinical significance. The valves functioned competently in all instances, with no regurgitation or stenosis at the valvular orifice.

SUMMARY

This case of subacute bacterial endocarditis was confined to a pulmonic valve in which there was almost complete absence of two of the leaflets, the underlying etiology being congenital maldevelopment. Despite the marked insufficiency, there were no demonstrable changes in the myocardium of the right side of the heart, a fact partially explained by the compensatory action of a large pedunculated vegetation which functioned as a ball valve.

REFERENCES

1. De Candole, C. A.: Reports of a Case of Malignant Endocarditis Confined to the Pulmonic Valve, *J. Roy. Army M. Corps* 116-117, 1936.
2. Lehman, Robert G.: Acute Bacterial Endocarditis of the Pulmonic Valve, With Acquired Stenosis, *Ohio State M. J.* 4, 38: 345-346, 1942.

3. Leader, Sidney G., and Kugel, M. D.: Pulmonary Stenosis of Inflammatory and Developmental Origin Complicated by Rheumatic Heart Disease, and Subacute Bacterial Endocarditis, *J. Pediat.* 595-603, 1940.
4. Grayzel, David M.: Vegetative Endocarditis of the Pulmonic Valve, *Yale J. Biol. & Med.* 7: 515-520, 1935.
5. Cockayne, E. A., and Wilton, T. N. D.: Acute Bacterial Endocarditis—Two Unusual Cases, *Lancet* 728-729, 1941.
6. Allen, A. C.: Mechanism of Implantation of Bacterial Vegetations in the Heart, *Arch. Path.* 27: 3; 408, 1939.
7. Libman, E.: Characteristics of Various Forms of Endocarditis, *J. A. M. A.* 80: 813, 1923.
8. Denman, Harold C.: Review of 50 Cases of Subacute Bacterial Endocarditis With Autopsy Findings, *Ann. of Int. Med.* 5: 904-919, 1942.
9. Thayer, Wm. S.: Studies on Bacterial Endocarditis, Baltimore, Md., 1926, Johns Hopkins Press, 186 p.
10. Clawson, B. J., and Bell, E. T.: Comparison of Acute Rheumatic and Subacute Bacterial Endocarditis, *Arch. Int. Med.* 37: 66-81, 1926.
11. Abbott, Maude E.: McGill University Exhibit: Development of Heart and Clinical Classification of Congenital Cardiac Disease, *Brit. M. J.* 2: 1197-1199, 1932.
12. De Vries, J.: Virchows *Arch. f. Path. Anat.*, pp. 91-193, Berlin, 1883.
13. Koletsky, Simon: Congenital Bicuspid Pulmonary Valves, *Arch. Path.* 31: 338-353, 1941.
14. Simonds, J. P.: Congenital Malformation of the Aortic and Pulmonary Valves, *Am. J. M. Sc.* 166: 584-595, 1923.
15. Bishop, L. F., and Trubek, Max: A Differential Study Between Inflammatory and Congenital Origin Bicuspid Aortic Valves, *J. Tech. Methods* 15: 111-131, 1936.
16. Koletsky, Simon: Congenital Bicuspid Aortic Valves, *Arch. Int. Med.* 67: 129-156, 1941.

A NOTE ON A POSSIBLE ALLERGIC FACTOR IN ALTITUDE SICKNESS

JULIA BAKER, M.D., MEXICO CITY

EVIDENCE has been accumulated in Mexico City (altitude 7,325 feet) which indicates that allergic reactions are more common at that altitude than at lower altitudes. From this I have deduced *Corrolary No. 1*: Mountain sickness, symptoms of which are the same as those of allergic reactions, may be explained on an allergic basis, and *Corrolary No. 2*: The higher the altitude, the more allergy will be encountered. Therefore, everyone may be potentially allergic and may develop some type of allergy if he stays at a high enough altitude long enough. The importance and practical application of this is that aviators flying at high altitudes may increase their efficiency and avoid unpleasant reactions by taking precautions used in allergic cases.

These points are to be supported by case histories and data on 500 cases 15 years of age and under, arranged in alphabetical order and taken from my files without selection of any kind. The cases were about equally distributed as to sex. There were 350 American or European and 150 Mexican patients.

In support of the theory that allergic reactions are more common at higher than at lower altitudes, I shall present data regarding hives and urticaria, and regarding gastrointestinal symptoms.

As a basis for comparison with my data, I have chosen the experiences of Vaughan¹ summarized in his statement: "I believe that we shall eventually find ourselves within striking distance of the actual facts when we accept the statement that approximately 10 per cent of the population suffers from frank allergy, major allergy, while an additional 40 or 50 per cent have experienced or will experience some minor evanescent allergic manifestations, not sufficiently pronounced to require medical consultation."

I. Hives, Urticarial Rashes and Eczema.—

Out of 500 individuals, 167 had either severe hives, urticarial rashes or eczema, there being 25 cases of the latter.

The diagnosis of hives was made on the basis of (1) appearance, (2) location and the ruling out of insect bites, (3) the proved relation to taking certain foods three or more times and improvement on omitting them three or more times, (4) the occurrence often coincident with gastrointestinal upsets. The diagnosis of eczema was made on the basis of characteristic appearance and location usually in antecubital and popliteal spaces.

II. Gastrointestinal Disturbances: Diarrhea, Nausea and Vomiting.—

In this group many things must be considered in differential diagnosis. These cases are not so obviously allergic on first inspection as hives and eczema. However, the following points are considered in ruling out other conditions:

1. Low blood count.
2. Negative stool cultures.

3. Relation to certain foods: giving certain foods causes symptoms, and removing these foods causes improvement.

4. Relation to hives and urticaria. rashes, both gastrointestinal disturbances and skin conditions often occurring simultaneously.

Of the 500 cases, 100 had diarrhea, vomiting accompanying this in 48 individuals. The most common offenders were eggs, milk, orange juice, and chocolate in that order. Symptoms in most of these cases became acute within three weeks of arrival in Mexico City. Later, symptoms are still present but in a more chronic form such as anorexia, failure to gain weight, fatigue, irritability. These symptoms clear up on finding and removing the offending foods from the diet. These reactions are definitely not due to some specific abnormality of food or water in Mexico City, as many children develop hives, diarrhea and vomiting traceable to food products imported from the United States such as cod liver oil, canned evaporated or powdered milk. These cases clear up when the particular food is omitted from the diet or when the patient goes to a lower altitude where he may take that food.

CASES

B. B., female, born April, 1940. Sept. 6, 1941. Sudden urticaria on back, right arm fingers and legs, following breakfast of Pablum, orange juice, milk and soft boiled egg. Eggs were suspected, but four foods were omitted from the diet: orange juice, tomato juice, chocolate and eggs. This restriction plus enema and laxative resulted in prompt subsidence of urticaria. Oct. 24 to Jan. 3, 1942, the patient was in Los Angeles, Calif., and there ate oranges and eggs without any difficulty. On Mar. 26, 1942, the child had had severe itching urticarial rash all over body for five days, getting worse. She had been taking egg and orange juice since her return Jan. 3, 1942. Treatment was enema, laxative, and omission of orange juice and eggs. The rash began to subside the next day. Apr. 14, 1942. Her mother reported that eggs without orange juice caused a rash, and that orange juice given alone caused no trouble.

B. B., male, born June, 1939. Aged 4 years. He has mild eczema in Mexico City and not in Indiana. He can take fresh cow's milk and evaporated milk in Indiana, but can take only small amounts of condensed milk here.

A. A. B., born June, 1937, and seen first Aug. 10, 1940, soon after arrival in Mexico City, because of vomiting. Physical examination gave no explanation for vomiting and temperature was normal. Urino was +++ for acetone. On Sept. 30, 1940, the patient had another attack of vomiting which lasted only one day. On Oct. 15, 1940, she was vomiting again, and was advised to omit eggs from the diet. By Dec. 21, 1942, the child's mother had repeatedly observed that trouble was related to ingestion of egg, and that the child has decided reaction even to small amounts. She had never had any such trouble before coming to Mexico City.

P. C., male, born May 24, 1935. Oct. 16, 1942. Patient was first seen shortly after arrival in Mexico City because of vomiting, and was found to be acidotic (acetone ++). Vomiting continued, lactate-Ringer's solution was given intravenously with prompt recovery. Advice was given regarding foods liable to cause trouble at this altitude. On January 10, 1943, Feb. 5, Mar. 24, July 9, and August 28, 1943, this boy was seen because of abdominal pain and nausea and severe hives. These attacks always responded to temporary omission of milk, eggs, chocolate and orange juice. These foods were later tolerated, but only in small amounts. He had never had any hives or gastrointestinal disturbances from these foods in New York City. He went to New York and stayed from September to November, 1942, and ate everything without having hives or abdominal pain.

J. D., male, born June 25, 1930. November, 1939. Boy was seen first due to severe hives with secondary infection. By means of carefully followed elimination diet, eggs were found to cause hives on three occasions and were then omitted from the diet. Chocolate was

found to cause abdominal pain, nausea and vomiting, and later hives on various occasions, but was eaten at parties from time to time. On trips to Acapulco (sea level) he ate these foods daily without trouble. In September, 1943, he went to Boston to boarding school, where he was advised to try these foods and note results. He reported in February, 1944, that he can eat everything and has no trouble with hives or digestive difficulties.

T. E., female, born September, 1938. At three months of age, this patient developed a rash and vomiting. She was taken by plane to Brownsville (sea level), a three hours' trip, and recovered immediately on arriving at sea level. She goes to Brownsville for two months every six months and eats all foods, without any hives or gastrointestinal disturbance. Here in Mexico City by keeping a diet record it has been found that orange juice, chocolate, egg, and milk if taken in too large quantity, all cause hives, and the hives subside on omitting these foods. This has been recorded on the written record various times.

D. F., female, 13 years old, was seen a few days after arrival in Mexico City because of infected hives, abdominal pain, and diarrhea. She had never had hives in England where she had lived before coming here. The hives began on arrival and became worse until she omitted orange juice, chocolate, eggs, and milk from her diet when they cleared up. The exact food involved was not ascertained, as she left shortly after this.

J. G., female, born July 31, 1936, has hives frequently here. These clear up completely in Brownsville and Acapulco. Skin tests in 1939 were positive for oatmeal and rice. In 1943, thirty scratch tests were negative except oatmeal. Rice in spite of being negative has caused a cough on three different occasions. Oranges cause a cough and running nose. These foods were taken without trouble in Brownsville.

S. P., female, born July 17, 1939, was seen July 21, 1941, because of infected hives. She had had hives since 1½ years of age. She was put on an elimination diet, foods being added gradually. On Sept. 18, 1941, skin tests showed egg slightly positive, wheat negative. Nov. 2, 1942. History to date showed that oatmeal caused hives immediately, and that chocolate given for three days caused hives. Eggs caused hives within one day on various occasions. Emotional upsets were observed related to hives, and temper tantrums and hives often occurred together. Feb. 10, 1944. Foods that cause trouble here (eggs, chocolate, peanuts, oatmeal) have been given in Acapulco without any trouble on three different trips. Eggs are given every day in Acapulco with no ill effects. The child breaks out in hives on returning to Mexico City.

C. P., male, born April 21, 1937. This child has had abdominal pain and vomiting related to eggs on four occasions, with prompt subsidence of symptoms on omitting egg from diet. He can eat eggs every day in Acapulco.

T. P., born 1937. This patient was first seen June 17, 1942, at the age of five and a half years with the chief complaint of hay fever especially after his afternoon nap. He has hives here but not in Los Angeles, California. From October, 1941, to May, 1942, he has hives here and free from hay fever, sneezing, and hives, which are frequent complaints in Mexico City. Intradermal skin tests showed marked reactions to both feathers and house dust, with orris root negative. The mother was advised to cover the pillow with waterproof dust, with orris root negative. On June 24, after covering the pillow, he had improved and keep a diary of contacts. On June 24, after covering the pillow, he had improved so that he no longer had sneezing or running nose after his nap, but there still persisted some inflammation of the conjunctivae. Tuberculin test was negative and blood count was as follows: White blood cells 4,750, red blood cells 4,780,000, hemoglobin 78, polys 62, stabs 5, lymphs 20, monos 13. Oct. 19, 1942, his mother reports that he is having hives only every three to four weeks. On Dec. 27, 1942, she reported that oranges were known to cause hives on several occasions. On Oct. 6, 1943, the boy had diarrhea and severe hives, which cleared up with dietary restrictions.

S. P., male, born Mar. 6, 1936, and seen first Sept. 5, 1940, shortly after arrival, because of loose bowel movements, from three to four daily for four days. He recovered promptly on a diet of rice gruel, tea, and gelatin. On Oct. 12, 1940, he had vomiting and abdominal pain preceded by several loose bowel movements and running nose for several days. Pain was intermittent but severe, abdomen showed slight splinting, and rectal examination was painful. At noon the blood count was W. B. C. 6,550, polys 48, stabs 18, lymphs 24, monos 10. At 6 P.M. the blood count was W.B.C. 9,125, polys 32, stabs 16, lymphs 40, monos 12. At 9 P.M. the boy was much better and on Oct. 13, the temperature was normal and he had no

pain. On December 8, 1940, he had vomiting without fever. Since arrival the boy had had frequent abdominal pain occurring about every week, and colds every month lasting from one to two weeks; therefore he had been half sick most of the time. Dec. 11, 1940: Patient vomited five times during the night, and had no fever. Dec. 12, 1940: He was put on a Rowe elimination diet, and foods gradually added. Jan. 2, 1941: Progress was reported as good. Feb. 3, 1941: His mother said that he had had six weeks without vomiting, pain, or running nose. His hemoglobin at this time was 11.5 Gm. June 17, 1941: Summary from diet records showed that he had along very well except when eating chocolate, egg or orange juice. Sept. 8, 1941: The child had vomiting without fever. He had eaten both egg and orange juice, fed by a new servant. Nov. 17, 1941: Patient had asthma for the first time. Nov. 25, 1941: Patient was tested intradermally with eight inhalants and found to have a marked reaction to cats, and a moderate one to kapok and feathers. Dec. 1, 1941: Patient developed mumps, and recovered normally. Mar. 1, 1942: Patient had a severe spell of vomiting without fever, acetone in urine ++, considered due to eating chocolate. Apr. 21, 1942: First dose, 1 c.c., of tetanus toxoid was given. Apr. 22, 1942: The boy developed severe asthma and was given adrenalin. May 7, 1942: Asthma recurred, but was less severe. Intradermal tests for feathers and house dust were repeated, and the reactions were considered to be sufficiently increased to warrant desensitization which was begun and carried out until he left in July, 1943. May 27, 1942: The boy was beginning to gain weight and his nose had stopped running. June 9, 1942: He began to lose weight again. July 10, 1942: Patient had vomiting, headache, sneezing, and nose running copiously. On July 14 he was taken off of milk and Aug. 7 had gained some weight again. August 17 he had a mild attack of asthma. Sept. 10, 1942: The boy had asthma developing after a bad cold and cough of five days' duration. He had a marked reaction to ragweed. On Oct. 19, 1942, he had sneezing and coughing, and no more trouble until Dec. 2, when he had mild asthma and running nose, with slight hyperemia of the right tympanic membrane, and 101.7° F. Recovery was uneventful.

Until April 15, 1943, he had a period of improved health, with, however, no gain since September. At this time he drank chocolate and on April 16 he had severe headache, nausea and running nose. Enema and milk of magnesia were given and on April 17 he was better. Throughout the history there has been considerable difficulty getting the servants to observe the diet restrictions. On April 22, 1943 he had asthma and running nose, and on May 10 running nose and sneezing. It has been noted that after two or three parties where sandwiches and cake are served, i.e., large amounts of wheat, that he invariably gets a headache. On June 13, 1943 he went to a picnic, ate much bread, and on June 14 had headache, stomach-ache, and temperature 102° F. An enema was given and on June 15 his temperature was normal and he was feeling fine. July 10, 1943: Family left for Quantico, Va. On Dec., 1943, his mother reports that the boy can eat all foods including chocolate without discomfort of any kind, and that he has been in good health with no recurrence of the asthma or headache and vomiting. In other words, he had not had these symptoms before coming to Mexico City; they were marked during his stay here of nearly three years, and he did not have them after returning to sea level.

M. P., male, born June 9, 1939, younger brother of preceding case, was seen first Feb. 3, 1941. On Feb. 27, 1941, he had roseola infantum and on June 17, 1941, a scarlatiniform rash on body, slight amount on face and none on the extremities, without fever. On Sept. 4, 1941, he had hives. On April 1, 8, and 15, 1942, he was given typhoid vaccine. April 27, 1942, he developed cough and running nose, at which time intradermal skin tests were done with a marked reaction to orris root, a mild reaction to feathers and a negative reaction to house dust.

On May 8, 1942, he had severe hives and was placed on an elimination diet, which however was not carefully followed. On May 15 he had more hives and on May 19 the hives continued and he had a running nose. On June 9 he had a mild attack of asthma. Twenty scratch tests were done, including both foods and inhalants. He had positive reactions to milk, rye, peas, horse and goat dander, orris root, kapok and feathers. By June 16 he was better, and he was given the second tetanus toxoid injection on June 23 with no ill effects. He continued to improve and by July 12 evidence indicated that bread caused the hives. The rest of his history is uneventful except for an upper respiratory infection in October with follicular

tonsillitis from which he recovered normally, frequent complaints of stomach-ache in January, 1943, and a cough and running nose in April. This boy got along better than his older brother, S. P. He was considered to be potentially allergic and measures were instituted to protect him, from the beginning, by means of a dust free room, avoidance of foods commonly causing trouble, and varying the diet as much as possible. However his condition was not ideal, and he had allergic reactions from time to time until going to Quantico, Va., where he had none in spite of eating everything, and taking no particular precautions. Like his brother, he had been well until coming to Mexico City, and was well again after going to sea level.

P. C. S., female, born July 31, 1936, and seen shortly after arrival from sta. Engracia (altitude 800 feet) on Aug. 11, 1942. This child had never had hives before coming to Mexico City; they began about two weeks after arrival, and were accompanied by anorexia. On examination a running nose, inflamed conjunctivae and severe hives were found. She was put on an elimination diet. By Aug. 18 the skin was clearing, but the conjunctivae were still inflamed. By Sept. 11 enough evidence had been collected to indicate that tomatoes caused the hives and this was later corroborated by a report on Oct. 20, at which time the skin was clear except for a small patch of eczema on the left cheek and eyelid. She had a positive reaction to feathers and was advised to have her pillow covered with waterproof material. She was seen again Jan. 22, 1943, at which time the conjunctivae were better and it was reported that she had had no trouble in the interval.

J. S., female, born Sept. 2, 1941, was 9 months old when first seen, with a history of never having been ill. She arrived in Mexico on June 2, 1942, and became ill June 6 with fever and ten bowel movements in twenty-four hours. The blood count was 9,600 W. B. C., 28 polys, 17 stabs, and 55 lymphocytes, and the stool culture was negative. On June 8 the count was W. B. C. 6,800, polys 28, lymphs 49, monos 6, and stabs 17. Sulfaguanidine was given, to which the response if any was slow. She was given powdered lactic acid milk, gradually increasing from a weak formula. On July 10, 1942, she had four loose bowel movements which were considered due to liver soup.

On August 1 she again had diarrhea, eight bowel movements in twenty-four hours. No treatment was given except to take her off milk and in 48 hours her bowel movements were two per twenty-four hours. She was then given Sobee which agreed with her for about two weeks. On Aug. 16 she lost her appetite, and had a running nose and cough. Otitis media developed on Aug. 20 and she had fever and a slight rash. She was started on sulfadiazine and nose drops. There was no improvement, the fever and rash continuing, to which was added marked flatulence. On Aug. 24 Sobee was discontinued, and on Aug. 26 the baby had normal temperature and the rash had disappeared. Urine was negative, and W. B. C. 10,750, polys 13, lymphs 87. By Aug. 27 she had a good appetite and her cold had entirely cleared up, W. B. C. 13,050, polys 13, lymphs 86, bas. 1. In September she had fever and a running nose for two days. Urine was negative. In October she had diarrhea again with fever of 102.5° and vomiting. There were 9 bowel movements in the first twenty-four hours, on Oct. 11. This cleared up on a restricted diet. On Oct. 14 in spite of a normal temperature she had nausea and vomiting for one day. The urine was negative and the blood count was as follows: W. B. C. 10,300, polys 21, lymphs 77, monos 2, hemoglobin 14.5 Gm. Her health was good until Feb. 6, 1943, when she had a severe but short illness, with fever ranging between 104° and 105° F. for two days. The physical examination was negative except for eczema in the popliteal spaces. Urine was negative, chest x-ray was negative and there was no blood or pus in the bowel movement. The blood counts are given below:

Feb. 6	W. B. C. 27,750	polys 46	stabs 22	lymphs 29	monos 3	
8	W. B. C. 32,750	polys 54	stabs 14	lymphs 49	monos 3	
9	W. B. C. 11,800	polys 17	stabs 11	lymphs 70		Eo 2
10	W. B. C. 7,350	polys 16	stabs 1	lymphs 81	monos 1	Eo 1

In view of her history, after finding no evidence of infection, every effort was made to clear out her gastrointestinal tract by means of frequent enemas and milk of magnesia. Aspirin did not bring down the fever during the first day and she had one convulsion. Sulfadiazine was given, 1 grain per pound per day, and a blood culture was considered but was not done when the temperature began to drop quickly and stay down. On Feb. 9 the child had a normal temperature and was feeling well. On February 5 the child had had corn starch

pudding made with milk, two large helpings. This was the first milk she had had since August, 1942. No explanation was found which could adequately explain this illness.

Shortly after this she was taken to Los Angeles, Calif., where she took all foods including milk and had no trouble or illness. She was advised to do this, but five days before return to Mexico City she was to go on a diet omitting the foods known to cause trouble here, i.e., lamb, liver, eggs, and milk. On May 4, 1943, she returned from California. She had no trouble until May 22, when she had a temperature of 104.5° F., and much fetid gas. On May 21 she had eaten cakes made with milk and egg, the first time she had broken the diet since her return. The blood count was: W. B. C. 15,850, polys 35, stabs 3, lymphs 58, monos 4. Treatment was milk of magnesia and enema and on May 23 she was well. Desensitization to milk was begun very gradually in June, 1943. Wheat was still omitted because of activating the eczema. In December, 1943, the child could take milk, but not wheat. Her condition at this time was good.

M. S., female, born February, 1932, and seen first Feb. 24, 1941. There was a history of colds about every month, recurrent otitis media about fifteen times with mastoid involvement twice. Teeth were carious. Tuberculin test was positive, and chest x-ray negative. Tonsillectomy was done at 18 months of age. The only history of allergy was occasional hives resulting from "cold" injections. Physical examination showed a pale, undernourished nine-year-old girl. On March 10 hemoglobin was 14 Gm., W. B. C. 11,100, R. B. C. 4,930,000, polys 40, Eo 8, lymph 50, monos 2.

April 24, 1941. Stool examination showed cysts of *Endomeba histolytica*. Treatment was with iodoform and carborsone, after which stools were negative. July 16, 1941. Patient was seen because of vomiting and hives, no fever. This subsided on the usual treatment of enema, milk of magnesia, and temporarily restricted diet. On Sept. 30, 1941, sedimentation rate was normal and hemoglobin was 15.5 Gm. On Oct. 14, 1941, she had asthma for the first time. Skin scratch tests of 17 inhalants showed 23 foods negative, and cat hair, feathers and house dust markedly positive. Treatment was by means of elimination diet and dust-free room, with plans to desensitize her to feathers and house dust on her return from Acapulco. She improved on this regime. From Nov. 25 to Jan. 28, 1942, she was in Acapulco, sea level, where she ate everything, wheat, eggs, etc. Asthma developed on the trip up to Mexico City. On Feb. 10 she again had asthma, which was thought related to exposure to feathers, and she was advised to have the pillows covered. This was done. The asthma recurred Feb. 21, 23, and 28, but was milder. On Mar. 12 she had asthma and a cough as well, so on March 16, 1942, desensitization to feathers and house dust was begun and continued to date because of the fact that much of the furniture in the house is upholstered with feathers. On Apr. 11 she had a pain in her hip, back and shoulder without fever or swelling. April 16. Patient began eggs. On Apr. 23 she began to vomit, and had no fever. She was given an enema and milk of magnesia and told to omit eggs. The next day she was slightly better although feeling very weak, and on Apr. 25 she was well.

May 5, 1942. Typhoid injection caused temperature of 100.4° F. and malaise for three days. Other doses were given in divided amounts. The diet record showed that grapes had caused hives on several occasions. Her general condition was improving. On June 25, she had vomiting again without fever. After an enema, milk of magnesia, and omission of the latest addition, lamb, to the diet, she recovered and on June 26 was well. On July 4, 1942, she broke the diet and had hives the next day, and asthma on July 6. Raisins and grapes were known at this point to cause anorexia and hives. On October 25, 1942 she had sneezing and mild asthma which cleared on omitting milk.

From Dec. 5, 1942, until Feb. 10, 1943, she was in Acapulco for school vacations. She had no trouble going down but on coming up her nose began to run, with severe sneezing, which developed into asthma on arrival in Mexico City. She was advised to take an enema, milk of magnesia, and go back to the original diet for a few days. On Feb. 11 she was well and went to school. From Feb. 11, 1943, she has had regular desensitizations and diet restrictions except when in Acapulco, and has had no illness except influenza Nov. 18, 1943. From Dec. 3, 1943, until February, 1944, she was in Acapulco and ate all foods without restrictions or ill effects. She had no trouble going down but on coming up she had a running nose and sneezing.

E. S., female, born Mar. 29, 1941, began to refuse her milk Oct. 14, 1941. In November she was still taking less milk and losing weight. Sobee was tried but she would not take it. In January, 1942, she developed eczema on her elbow which improved with tar ointment. In April she had a mild diarrhea with green bowel movements, three in twenty-four hours; this cleared up with a temporarily restricted diet. On May 4, 1942, she had a running nose. July 7, 1942. The baby developed a patchy rash on the buttocks which was thought to be due to Monilia. The diapers were boiled, and gentian violet was used. This gave no results so modified Whitfield's ointment was tried. Treatment was given over three weeks during which time the condition was getting worse. On Aug. 6 the mother was told to omit milk and wheat and two days later the skin was nearly normal, and continued to improve. On Aug. 18 the baby was getting Sobee, but this was discontinued on Aug. 21 and Klim was tried again. On Aug. 26 the baby had a running nose, anorexia, was very fussy and had not gained weight. On Aug. 27 she was better, Klim having been omitted and an enema given, and on Aug. 28 she was much better.

On Oct. 28, 1942 she had vomiting and three bowel movements, and had had no appetite for five days. At this point the baby was taken to Brownsville for two months and there had no trouble at all in spite of taking all foods, and drinking milk. She was advised to go on the restricted diet five days before her return; this was done. However, her mother felt that since she could take milk in Brownsville so well she would try a little here. She gave evaporated milk; this produced urticaria. Several days later, Feb. 27 and 28, Klim was given, and she developed vomiting, acidosis, and temperature of 101° . She was still vomiting Mar. 1, and had a generalized urticaria involving face, body, and extremities, which was severe. Her temperature went to 102.2° on Mar. 2 and became normal on Mar. 3. Treatment was the usual one of clearing out the gastrointestinal tract, and intravenous lactate-Ringer's solution. Blood counts were as follows:

Mar. 3 W. B. C. 15,400, R. B. C. 4,000,000, Hb 11 Gm., polys 43, stnbs 4, lymphs 40, monos 9, Eo 3, B 1

9 W. B. C. 10,600, Hb. 12 Gm., polys 30, lymphs 65, monos 3, Eo 2

No milk, eggs, orange juice or chocolate were given from then on, and vitamin C and calcium were added. The mother was very careful to vary the diet, alternating foods so that no one food was given more than once or twice a week, and no food was taken in any great quantity. On this regime the baby improved steadily and gained weight.

On Nov. 11, 1943, she went to Brownsville and her mother was instructed to give foods omitted here, but to begin the Mexico City diet five days before return. On Feb. 18, 1944, her mother reports that in Brownsville she is taking pasteurized cow's milk, egg, bread, orange juice, in fact a diet without any restrictions, that she has been in excellent health and has gained seven pounds.

B. F., male, born Mar. 4, 1942, had had no illness before coming to Mexico. He was seen shortly after arrival Oct. 1, 1942, because of a running nose. On Oct. 21 he began to vomit, and his mother was advised to omit egg and orange juice from the diet, and to continue with evaporated milk. As he vomited frequently on Oct. 28, he was changed to Klim. Nov. 2 he had a running nose again and Nov. 23 anorexia and loose bowel movements, two a day. On Dec. 3 this developed into diarrhea with six bowel movements a day, which however were not liquid. On omitting all milk, the diarrhea cleared up. Dec. 23 he had another spell of running nose, and the same on Jan. 15, 1943. On Jan. 21 he took milk half strength for the first time since December and cried from 1 to 7 A.M. with colic and fever. Then he began to vomit and became acidotic, urine being positive for acetone and negative otherwise. Intravenous lactate-Ringer's solution was given, and the gastrointestinal tract was cleared out. On Jan. 23 he was well, and was started on a milk substitute of cereal, gelatin and oil, which he took well. On Feb. 10 he had his second combined diphtheria and tetanus toxoid injection with no reaction. On Feb. 18, 19, and once the morning of Feb. 20 he was given milk. He developed fever, vomiting and acidosis, and was treated with milk of magnesia, enemas, and subcutaneous fluids. The urine became free of acetone on Feb. 23. His mother was advised to give no milk of any kind for several months. On Apr. 10 he had fever, which lasted three days. Tuberculin test was negative. On Apr. 13 a Schick

test was done. He got along very well until May 18, when milk was given by mistake. His temperature went to 103° F., the fever lasting one day. Treatment was milk of magnesia and enema. On May 26, desensitization to milk was begun by giving very small amounts and gradually increasing. He was not able to take this, but could take boiled goat's milk on which he gained weight. In June, 1943, he went to Laredo, and his mother was advised to feed him everything while there, but to give the Mexico City diet three days before return. He took orange juice, eggs, evaporated cow's milk while there and was very well during the eighteen days. Shortly after his return in July he had three days of unexplained fever, and from then on was very well except for influenza in December, 1943.

A. W., female, born Sept. 11, 1939, was seen Sept. 30, 1943, four days after arriving in Mexico City from West Virginia. Hives began Sept. 28 and got progressively worse. She was treated with enema, milk of magnesia and omission of milk, eggs, orange juice and chocolate from the diet. On Oct. 1 she was better. On Oct. 7 she took orange juice and did not develop hives. On October 13, she ate eggs and had hives. On Jan. 7, 1944, she was in for a check up, and her mother stated that four times hives have been noted directly related to the ingestion of eggs. This child had never had hives before coming to Mexico.

D. Y., a 15-year-old girl was seen first Feb. 21, 1944, because of severe hives and angioneurotic edema. She had lived in Chicago, came down by train to the border and at Brownsville took the plane to Mexico City. She had never had any hives, hay fever, eczema or other allergic complaint in her life, though her father had eczema and her uncles hay fever. Within half an hour of travelling in the plane she was itching all over and by the time she arrived here, both eyes were swollen shut. Since arrival Dec. 16, 1943, she has been much troubled with hives and swelling, except when she went to Acapulco and got complete relief. On her return to Mexico City the hives and angioneurotic edema recurred. She was put on an elimination diet by another physician, but has not had time to arrive at conclusions as to what is causing the trouble. She is going to Acapulco again today, Feb. 22, 1944.

In addition to these cases, I should like to quote the statement of Dr. W. L. Garnett, U. S. Public Health physician here in Mexico City who says he frequently has adult cases, tourists to Mexico City, who complain of severe hives and who have never had hives before in their lives.

The high incidence, the accumulative period, and the character of the symptoms of these proved allergic manifestations in Mexico City are similar to those of mountain sickness. In that condition the well-known symptoms are nausea, vomiting, depression both mental and physical, and diarrhea. Moreover, experimental exposure to reduced partial pressures of oxygen has shown an accumulative period before symptoms appear.² At a four-hour daily exposure to a simulated altitude of 12,000 feet, mild symptoms appeared during the third week. With a daily exposure of seven hours, nausea, loss of appetite, etc., appeared at the end of the first week.

It has occurred to me that the anoxia experienced at an altitude such as that of Mexico City may result in a greater absorption and accumulation of protein products than at low levels. Something of this character may account for the very much higher incidence of allergic manifestations in Mexico City. In mountain sickness itself, similar factors may be possible contributing causes. Low partial pressure is evidently the basic cause of mountain sickness and this definitely modifies various physiological functions.

I have been able to find only one reference in the literature available to me indicating an influence of altitude on allergic manifestations. This is a paper by Kopaczewski and Marczewski³ on anaphylactic shock, recording the production of convulsions in guinea pigs sensitized to beef serum albumin one month previously. These animals together with normal controls were placed in a

pneumatie eisson at the Bourget airdrome and the atmosphere depressed at a rate corresponding to a rise in the air of 1,000 meters per minute until the equivalent of 10,000 was reached. At the latter figure all sensitized animals were in convulsions (with the exception of those which had gone into convulsions earlier). A slow descent was made. At 6,000 meters (in three minutes) all the animals slowly recovered, the only symptom persisting being a lowered temperature. The controls remained normal except for a slight lowering of temperature.

Loss in weight characteristically accompanies the anoxia of high altitudes and the reasons for this have been assumed to be loss of appetite, restlessness, diarrhea, etc.⁴ The loss in weight among members of mountain climbing expeditions has always been high. Of particular significance is the report of Hurtado⁵ that native Andean children in comparison with those of similar stock at sea level are underweight.

SUMMARY

From the records of a random sample from private practice of 500 individuals in Mexico City, fifteen years of age and under, about 50 per cent were found to be frankly allergic. Of the five hundred, 167 had either severe hives, urticarial rashes or eczema, and one hundred had gastrointestinal symptoms directly related to the ingestion of certain foods. Evidence indicates that these symptoms are either milder or do not occur at all in these same individuals at lower altitudes. In those coming to Mexico City, a period of about three weeks is usually required in the older children for the effect to accumulate and become manifest in symptoms. It is suggested that anoxia occurring at this altitude may result in a greater permeability of the gastrointestinal tract to offending substances. In view of the similarity in many of the cases to conditions found in mountain sickness, the possibility is suggested that similar factors may be contributing causes in such conditions, and may be of interest in aviation medicine.

Recommendations: Children with allergic reactions to foods do well at this altitude if the following precautions are taken: (1) avoiding overloading on any one food, (2) not giving any one food more than once or twice a week, (3) avoiding eggs, chocolate, and too much cow's milk, wheat and orange juice, and (4) supplementing the diet with vitamins A, D, B complex, C and calcium. If certain foods cause serious trouble, they must be temporarily omitted from the diet. Thus it is concluded that aviators who have symptoms similar to those described or others that cannot be explained except on an allergic basis would do well to take these precautions.

REFERENCES

1. Vaughan, Warren T.: *Practice of Allergy*, St. Louis, 1939. The C. V. Mosby Co.
2. Armstrong, H. G.: *Principles and Practice of Aviation Medicine*, Williams and Wilkins Co., Baltimore, 1939.
3. Kopaczewski, W., and Marczewski, S.: *Anaphylaxie du point de vue de l'altitude*, *Compt. rend. Acad. d. sc.* 201: (14), 569-570, 1935.
4. Van Liere, Edward J.: *Anoxia, Its Effect on the Body*, Chicago, University of Chicago Press, 1942.
5. Hurtado, A.: *Respiratory Adaptation in the Indian Natives of the Peruvian Andes. Studies at High Altitude*, *Am. Jr. Phys. Anthropol.* 17: 137-165, 1932.

EFFECT OF HEPARIN ON PHAGOCYTOSIS

OBSERVATIONS ON *P. LOPHURAE* IN THE CHICK

R. H. RIGDON, M.D., LITTLE ROCK, ARK.

EXPERIMENTAL observations have shown that heparin does not affect either the phagocytosis of staphylococci by polymorphonuclear leucocytes or the phagocytosis of particles of India ink by the cells of the reticulo-endothelial system.^{1, 2} Both of these studies were made upon the rabbit. Von Jansco³ observed that heparin when given intravenously to mice prevented the phagocytosis of colloidal particles of gold during an interval of ten minutes. Capell⁴ has emphasized a fact that is significant in any study of phagocytosis. That is "the reticulo-endothelial cells of the venous sinusoids are even more indifferent to particulate matter than to soluble dye." There may be a similar selectivity in the phagocytosis of various types of substances by cells of the different organs.

The mechanism of phagocytosis is not fully understood at the present time. Both normal and immune sera aid the process of phagocytosis of bacteria by leucocytes. Wright and Douglas⁵ gave the name "opsonins" to the active substance which in their opinion was present in serum and aided phagocytosis. It appears from previous experimental studies that heparin does not affect the relationship of serum to the process of phagocytosis of staphylococci by leucocytes.¹ Few observations have been made upon the effect of heparin on bacteriologic and immunologic processes.

Phagocytosis apparently is a significant process in the defense of the host against plasmodia. This process of phagocytosis occurs in all known species infected with malaria. Kinsely and his associates⁶ recently advanced the theory that malarial parasitized erythrocytes are coated with either fibrin or a fibrin-like substance and only these coated erythrocytes are phagocytosed. Furthermore, this fibrin or fibrinlike substance produces a clumping of red blood cells. These masses are phagocytized more readily than individual cells. Kite and Wherry⁷ in 1915 suggested "that foreign particles as carbon, carmin granules, etc., are "taken up" by leucocytes because the latter . . . have sticky surfaces."

This paper is a report of our observations upon the effect of heparin on the parasitemia produced by *P. lophurae* in chicks. In this study we concur in the opinion that malarial parasites are removed by a process of phagocytosis. This does not imply, however, that phagocytosis is the only defense mechanism that the body has against plasmodia. Observations are also included in this paper on the effect of heparin upon agglutination of both red blood cells and bacteria.

From the Department of Pathology, School of Medicine, University of Arkansas, Little Rock, Arkansas. Research Paper No. 545, Journal Series.

The data for this paper were obtained during the time I was a member of the Department of Pathology at the University of Tennessee, Memphis, Tennessee.

Received for publication, March 8, 1944.

METHODS AND MATERIALS

P. lophurae was the strain of malarial parasite used in these experiments. It had been transmitted through young white Pekin ducks at frequent intervals for approximately two years. Six months preceding the time that this experiment was begun, these parasites were transferred to young chicks and thereafter carried in young chicks.

The chicks with malaria were killed on the third or the fourth day of the disease. The blood was obtained immediately from the heart, mixed with an equal volume of a 2 per cent solution of sodium citrate in normal saline. One half cubic centimeter of this inoculum was injected intravenously into the veins about the ankle. The chicks varied in age from three to ten days. In each experiment, however, an identical group of birds was used for both the experimental and the control observation. A majority of the chicks were white giants. White leghorns and white rocks were also used.

The anticoagulant used in these experiments and referred to as heparin was "Liquaemin." It was supplied by Roche-Organon, Inc., through the courtesy of Dr. R. J. Floody. The following procedure was used: 0.1 c.c. of heparin was injected intramuscularly every four hours. A total of six injections was given. Heparin was injected into the chicks with malaria at different intervals as given in the individual experiments.

One cubic centimeter of blood was removed from the heart of the chicks immediately after they were sacrificed. This blood was placed in a test tube (7.5×1.0 cm.). It was observed to determine the time in which clotting occurred. The tubes were kept at room temperature for twenty-four hours. They were carefully inverted at intervals to determine the presence or the absence of clots. The blood from normal chicks clots within an interval of five minutes.

The degree of parasitemia was determined by counting the number of parasites in a given number of red blood cells. Blood for these smears was obtained from the toes. They were stained with a combination of Wright's and Giemsa's stains. The number of parasites in 100 cells was counted. These data were used for the statistical analysis. The number of parasites in 500 red blood cells was used for the graphs. This represents a multiple of five for the actual number of red cells counted. The points plotted upon the graphs represent mean values of the counts.

In the experiments where the effect of heparin on clotted blood was studied 1.0 cubic centimeter of blood was obtained from the chick's heart and placed in a test tube (7.5×1.0 cm.). After clotting, 1.0 cubic centimeter of heparin was added and the clot was separated from the wall of the tube by carefully rimming it with a small wooden stick. Tubes were kept both at room temperature and in an incubator at 37.5° C. for 24 hours. The clots were then examined microscopically.

In studying the effect of heparin on the agglutination of red blood cells, types two and three sera were used. A suspension of red cells was obtained from the same blood types. A series of titrations were prepared in which heparin plus an equal volume of saline, was used as a substitute for saline in the control. In a second group of observations, serum was placed on a

glass slide with a suspension of red blood cells of opposite type. After agglutination one to two drops of heparin were added. The heparin was added to the serum in some of these latter observations preceding the addition of the red blood cells.

In observing the effect of heparin on the agglutination of bacteria, an (H) typhoid antigen and immune serum were used. A series of dilutions, 1:10 to 1:10, 640 were prepared. Saline was used as the control diluent and heparin as the diluent in the experimental group. In one series the antigen was suspended in saline, and in a second it was suspended in heparin.

The "t" distribution was used for the testing of significance.*

EFFECT OF HEPARIN ON THE CLOTING OF BLOOD IN NORMAL CHICKS

A group of eight chicks was given intramuscularly two injections of 0.1 cubic centimeters of heparin at four-hour intervals. Two of these were killed four hours subsequent to the last injection. The blood from each of these failed to clot within 24 hours. Two chicks in this group were killed eight hours following the last injection. The blood from these clotted within a period of 30 minutes. Two chicks were killed 10 hours after the last injection of heparin. The blood from one of these chicks clotted within an interval of 10 minutes while that from the second chick clotted within a period of 20 minutes. The blood from normal chicks clots within an interval of five minutes.

TABLE I
EFFECT OF HEPARIN ON THE TIME OF CLOTING OF CHICK'S BLOOD*

NUMBER OF CHICKS KILLED	HOURS FOLLOWING LAST INJECTION OF HEPARIN	REMARKS
2	4	No clot formed within 24 hours
2	8	No clot formed within 24 hours
13	11	Clot formed in 30 minutes in 2 chicks Clot formed in 60 minutes in 3 chicks Clot formed in 24 hours in 2 chicks No clot formed in 24 hours in 6 chicks
2	12	No clot formed within 24 hours.
2	20	Clot formed in 2 minutes in 1 chick Clot formed in 10 minutes in 1 chick
4	36	Clot formed within 3 minutes

*0.1 c.c. of heparin injected intramuscularly every 4 hours for 6 injections. Chicks killed and 1.0 c.c. of blood withdrawn from heart.

A group of 25 chicks was injected intramuscularly with 0.1 c.c. of heparin every four hours for a total of six injections. These chicks were killed at subsequent intervals and the blood was observed to determine the time of clotting. The results of these observations are given in Table I. From these data it is evident that the time in which clotting occurs is delayed during a period of 12 hours following the last injection of heparin. In these chicks there is little if any variation from the normal in the time of clotting of the blood removed 20 hours following the last injection of heparin. Blood removed 36 hours subsequent to the last injection of heparin clots within the same interval as that from the controls.

*I am greatly indebted to Dr. F. L. Roberts, Professor of Preventive Medicine, University of Tennessee, for his assistance in the statistical problems concerned in this study.

A retraction of the clot was present, after 24 hours, in the blood of three of the four chicks killed 36 hours after the last injection of heparin. Retraction of the clot did not occur in any of the blood removed from the chicks given the heparin and killed at shorter intervals than 36 hours. In some of these experiments it was observed that the serum overlying the cells was coagulated and that the underlying cells were not clotted. Infrequently, the red blood cells and the serum formed a homogenous clot without ever showing any settling of the red blood cells.

EFFECT OF HEPARIN ON THE PARASITEMIA WHEN GIVEN IMMEDIATELY AFTER THE INJECTION OF MALARIAL PARASITES

Two groups of chicks were used. The injections of heparin were begun thirty to sixty minutes following the inoculation of the malarial parasites. They were discontinued after a period of 24 hours. The degree of parasitemia is given in Fig. 1. From these data it is evident that the number of parasites in the chicks given heparin is always less than that in the control. These data when analyzed statistically show a significant variation from the normal in the heparinized groups.

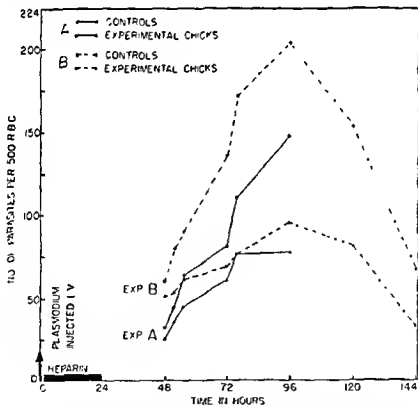


Fig. 1.—Effect of heparin on the parasitemia when given immediately after the injection of malarial parasites. In experiment A seven chicks were used in the experimental group and eight in the control. In experiment B eight chicks were used in each group. The heparin was given over a period of 20 hours in experiment B.

EFFECT OF HEPARIN ON THE PARASITEMIA WHEN GIVEN 24 HOURS FOLLOWING THE INJECTION OF THE MALARIAL PARASITES

In this experiment the parasites were given, and 24 hours later the injections of heparin were begun. They were continued for 20 hours. The degree of parasitemia is shown in Fig. 2. The number of parasites are less in the blood of the chicks given heparin than they are in the controls.

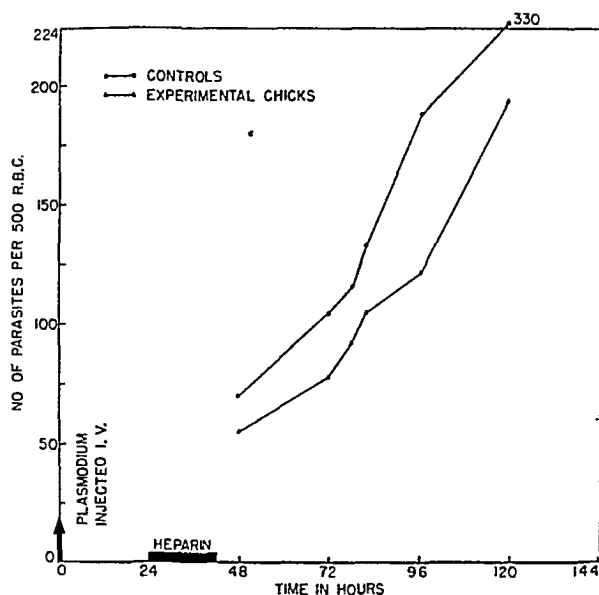


Fig. 2.—Effect of heparin on the parasitemia when given 24 hours following the injection of the malarial parasites. Ten chicks were used in the experimental group and seven in the control.

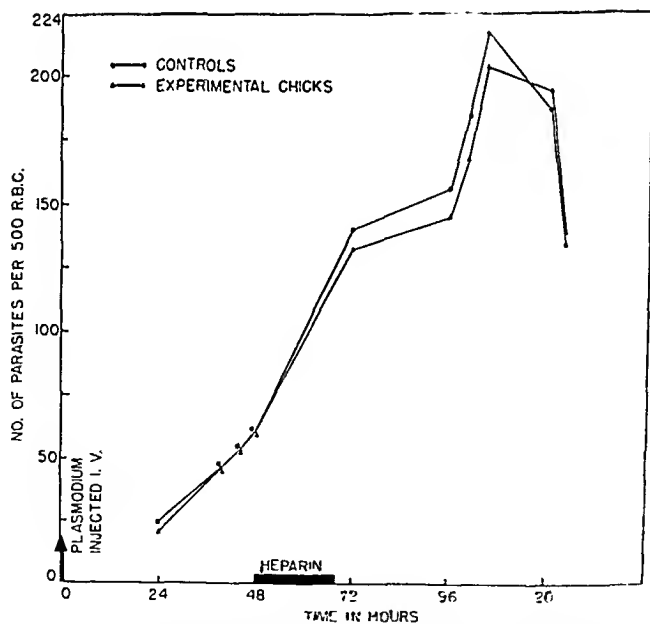


Fig. 3.—Effect of heparin on the parasitemia when given 48 hours following injection of malarial parasites. Fifteen chicks were used in the experimental group and ten in the control.

EFFECT OF HEPARIN ON THE PARASITEMIA WHEN GIVEN 48 HOURS
FOLLOWING THE INJECTION OF THE MALARIAL PARASITES

In this experiment the parasites were given intravenously, and 48 hours later the injections of heparin were begun. They were continued for 20 hours. The parasite count in these chicks is shown in Fig. 3. From these data there does not appear to be any variation in the parasitemia in the normal and heparinized chicks. These data when analyzed statistically do not show any significant variation in the heparinized group.

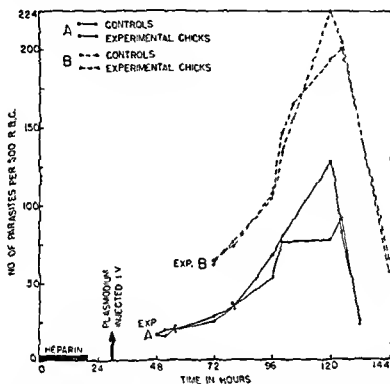


Fig. 4.—Effect of heparin on the parasitemia when given for 20 hours preceding the injection of malarial parasites. In experiment A six chicks were used in each group. In experiment B seven chicks were used in the control group and six in the experimental.

EFFECT OF HEPARIN ON THE PARASITEMIA WHEN GIVEN FOR 20 HOURS
PRECEDING THE INJECTION OF THE MALARIAL PARASITES

In the preceding experiment where heparin was given immediately following the injection of the malarial parasites the parasitemia was less than that in the control (Fig. 1). The results of that experiment suggest that the variation could be due to a direct effect of the heparin upon the chick. To study the effect of heparin on the parasitemia further, two groups of chicks were given heparin over a period of 20 hours. The parasites were inoculated 10 hours after the last injection of the heparin. The degree of parasitemia in these chicks is shown in Fig. 4. From these data there is no variation in the degree of parasitemia in the two heparinized groups.

EFFECT OF HEPARIN ON THE AGGLUTINATION OF HUMAN RED BLOOD
CELLS BY HETEROLOGOUS SERA

The exact mechanism of the agglutination of human red blood cells by heterologous sera is not fully understood. And, too, the mechanism of the effect of heparin on the clotting of blood apparently is not fully known. Since both of these phenomena may occur *in vivo* and also be produced *in vitro* the

effect of heparin on the former is included in our attempt to learn something of the effect of heparin on phagocytosis.

Serum, type 2 and 3, was titrated with heterologous red blood cells. The dilutions ranged from 1:10 to 1:640. There was no variation in either the degree or the extent of agglutination in the group of titrations containing both the heparin and the saline. Observations were also made in which the serum and the cells were placed upon a slide in the manner of the routine typing of blood. Heparin was added to the serum both before and after the addition of red blood cells. In no instance was the agglutination affected. Heparin was added to the agglutinated masses of red cells without producing any macroscopic change.

One may conclude from the present concept of the effect of heparin on the clotting of blood that this anticoagulant would not be likely to affect the agglutination of red blood cells. In the above experimental observation it is shown that heparin, *in vitro*, does not inhibit the agglutination of human red blood cells by heterologous sera.

EFFECT OF HEPARIN ON THE AGGLUTINATION OF TYPHOID BACILLI

In the preceding experiment it was shown that heparin had no effect upon the agglutination of red blood cells. In this experiment the effect of heparin on the agglutination of the (H) antigen of typhoid was studied. The dilutions of the titrations varied between 1:10 and 1:10,640. Heparin was substituted for saline in one series of these titrations. In a second, a dilution of heparin and saline was used. Saline was used for the control. There was no variation in either the degree or the extent of the agglutination in these three sets of titrations.

EFFECT OF HEPARIN ON CLOTS OF CHICKEN BLOOD

Blood was withdrawn from the heart of normal chicks and 1.0 c.c. was placed immediately in test tubes (7.5×1.0 cm.). This blood clotted within an interval of five minutes. One cubic centimeter of heparin was then added to the clotted blood. The clot was separated from the wall of the tube. There was no macroscopic variation after 24 hours in the consistency of the clots when heparin was added or in the controls.

The above procedure was repeated using blood from chicks with a high malarial parasitemia. Heparin, likewise, did not affect these blood clots. It is well known that heparin does not affect a formed thrombus. The present results are consistent, therefore, with similar observations of other investigators. Furthermore, they show that clotted blood, containing malarial parasites, is not affected by heparin.

DISCUSSION

The data in these experiments show that the parasite count is less in chicks given heparin than it is in the controls when the heparin is given within a period of 48 hours following the injection of plasmodia. Furthermore, the data suggest that heparin does not affect the parasite count when it is given in a similar quantity either 24 hours preceding or 48 hours subsequent to the injection of the parasites.

The probable error in this method of computing parasite density is very high. The time at which the counts were made vary in the different groups. Because of these facts the significance of a statistical analysis is limited. It is interesting to note in these data that the number of malarial parasites in chicks given heparin is always less than it is in the controls. There is insufficient evidence, however, at the present time, to determine, first, whether heparin stimulates phagocytosis; second, whether it has an injurious effect either directly upon the parasites, or in some other manner affects the body's defense mechanism against the plasmodium. It is suggested that heparin may affect directly *P. lophurae* since the greatest diminution in the parasite count occurs during the early phase of the infection. When the disease is present for 48 hours and heparin is given there is not a demonstrable variation in the parasite count.

Apparently there is nothing in the present study to indicate that the heparin inhibits the phagocytosis of malarial parasites. This observation may be significant in view of the conclusion made by Kinsely and his associates⁶ from their study of *P. knowlesi* infection in monkeys. They state that "a layer of fibrin or a fibrinlike substance is precipitated on knowlesi parasitized erythrocytes. Coated erythrocytes do not stick to ordinary endothelium but do stick to one another, forming "clumps" which stick to and are instantly engulfed by hepatic phagocytes. . . . Ordinary endothelium becomes sticky, too, and solidly coated with leucocytes. Hence leucopenia in drawn malarial blood." Rabinovitch⁸ has shown that heparin will prevent the formation of fibrinous thrombi in rabbits given dogs' serum while this anticoagulant does not affect the formation of agglutinated red cells which follow the injection of ox serum.

In the pathological study of the tissue from the same monkeys used by Kinsely and his associates⁶ Rigdon and Stratman-Thomas⁹ were unable to demonstrate "clumps" in the circulatory system, and likewise, they did not observe that the vascular endothelium was "solidly coated with leucocytes." If these "clumps," as observed by Kinsely, develop as a result of the parasitized erythrocytes being coated with either fibrin or a fibrinlike substance, it is obvious that something has occurred to this hypothecial substance that coats these erythrocytes because there were no clumps demonstrated in the sections. Quick¹⁰ has said "the theory that agglutination and phagocytosis depends on a fibrin coating about the affected particles or cells must include a mechanism whereby this fibrin is removed."

Thrombi and embolic masses from intravascular thrombi are readily demonstrable in paraffin prepared histological sections of tissue. By exerting gentle pressure on the lungs of rabbits that died following the injection of ox serum, Rabinovitch⁸ squeezed from the vessels numerous thrombi consisting of agglutinated red cells which were readily seen under the microscope. Only infrequently have masses been observed in blood smears from both man and monkey infected with malaria.^{11, 12} Best, Cowan, and McLean¹³ have discussed the mechanism of the formation of thrombi and they have shown that heparin inhibits their development. This anticoagulant has no effect upon a previously formed thrombus. Kinsely says that only parasitized erythrocytes coated with fibrin or a fibrinlike substance are phagocytosed. If so, it would appear likely that heparin may prevent the coating of these erythrocytes by fibrin and thus

effect of heparin on the former is included in our attempt to learn something of the effect of heparin on phagocytosis.

Serum, type 2 and 3, was titrated with heterologous red blood cells. The dilutions ranged from 1:10 to 1:640. There was no variation in either the degree or the extent of agglutination in the group of titrations containing both the heparin and the saline. Observations were also made in which the serum and the cells were placed upon a slide in the manner of the routine typing of blood. Heparin was added to the serum both before and after the addition of red blood cells. In no instance was the agglutination affected. Heparin was added to the agglutinated masses of red cells without producing any macroscopic change.

One may conclude from the present concept of the effect of heparin on the clotting of blood that this anticoagulant would not be likely to affect the agglutination of red blood cells. In the above experimental observation it is shown that heparin, *in vitro*, does not inhibit the agglutination of human red blood cells by heterologous sera.

EFFECT OF HEPARIN ON THE AGGLUTINATION OF TYPHOID BACILLI

In the preceding experiment it was shown that heparin had no effect upon the agglutination of red blood cells. In this experiment the effect of heparin on the agglutination of the (H) antigen of typhoid was studied. The dilutions of the titrations varied between 1:10 and 1:10,640. Heparin was substituted for saline in one series of these titrations. In a second, a dilution of heparin and saline was used. Saline was used for the control. There was no variation in either the degree or the extent of the agglutination in these three sets of titrations.

EFFECT OF HEPARIN ON CLOTS OF CHICKEN BLOOD

Blood was withdrawn from the heart of normal chicks and 1.0 c.c. was placed immediately in test tubes (7.5 × 1.0 cm.). This blood clotted within an interval of five minutes. One cubic centimeter of heparin was then added to the clotted blood. The clot was separated from the wall of the tube. There was no macroscopic variation after 24 hours in the consistency of the clots when heparin was added or in the controls.

The above procedure was repeated using blood from chicks with a high malarial parasitemia. Heparin, likewise, did not affect these blood clots. It is well known that heparin does not affect a formed thrombus. The present results are consistent, therefore, with similar observations of other investigators. Furthermore, they show that clotted blood, containing malarial parasites, is not affected by heparin.

DISCUSSION

The data in these experiments show that the parasite count is less in chicks given heparin than it is in the controls when the heparin is given within a period of 48 hours following the injection of plasmodia. Furthermore, the data suggest that heparin does not affect the parasite count when it is given in a similar quantity either 24 hours preceding or 48 hours subsequent to the injection of the parasites.

The probable error in this method of computing parasite density is very high. The time at which the counts were made vary in the different groups. Because of these facts the significance of a statistical analysis is limited. It is interesting to note in these data that the number of malarial parasites in chicks given heparin is always less than it is in the controls. There is insufficient evidence, however, at the present time, to determine, first, whether heparin stimulates phagocytosis; second, whether it has an injurious effect either directly upon the parasites, or in some other manner affects the body's defense mechanism against the plasmodium. It is suggested that heparin may affect directly *P. lophurae* since the greatest diminution in the parasite count occurs during the early phase of the infection. When the disease is present for 48 hours and heparin is given there is not a demonstrable variation in the parasite count.

Apparently there is nothing in the present study to indicate that the heparin inhibits the phagocytosis of malarial parasites. This observation may be significant in view of the conclusion made by Kinsely and his associates⁶ from their study of *P. knowlesi* infection in monkeys. They state that "a layer of fibrin or a fibrinlike substance is precipitated on knowlesi parasitized erythrocytes. Coated erythrocytes do not stick to ordinary endothelium but do stick to one another, forming "clumps" which stick to and are instantly engulfed by hepatic phagocytes. . . . Ordinary endothelium becomes sticky, too, and solidly coated with leucocytes. Hence leucopenia in drawn malarial blood." Rabinovitch⁸ has shown that heparin will prevent the formation of fibrinous thrombi in rabbits given dogs' serum while this anticoagulant does not affect the formation of agglutinated red cells which follow the injection of ox serum.

In the pathological study of the tissue from the same monkeys used by Kinsely and his associates⁶ Rigdon and Stratman-Thomas⁹ were unable to demonstrate "clumps" in the circulatory system, and likewise, they did not observe that the vascular endothelium was "solidly coated with leucocytes." If these "clumps," as observed by Kinsely, develop as a result of the parasitized erythrocytes being coated with either fibrin or a fibrinlike substance, it is obvious that something has occurred to this hypothecial substance that coats these erythrocytes because there were no clumps demonstrated in the sections. Quiek¹⁰ has said "the theory that agglutination and phagocytosis depends on a fibrin coating about the affected particles or cells must include a mechanism whereby this fibrin is removed."

Thrombi and embolic masses from intravascular thrombi are readily demonstrable in paraffin prepared histological sections of tissue. By exerting gentle pressure on the lungs of rabbits that died following the injection of ox serum, Rabinovitch⁸ squeezed from the vessels numerous thrombi consisting of agglutinated red cells which were readily seen under the microscope. Only infrequently have masses been observed in blood smears from both man and monkey infected with malaria.^{11, 12} Best, Cowan, and McLean¹³ have discussed the mechanism of the formation of thrombi and they have shown that heparin inhibits their development. This anticoagulant has no effect upon a previously formed thrombus. Kinsely says that only parasitized erythrocytes coated with fibrin or a fibrinlike substance are phagocytosed. If so, it would appear likely that heparin may prevent the coating of these erythrocytes by fibrin and thus

prevent them from being phagocytosed. The parasite count in chicks given heparin would be increased, therefore, over that of the controls. The data in these experiments do not indicate any inhibition in the degree of phagocytosis in the heparinized chicks.

It has been shown in previous experiments that heparin, when given intravenously to rabbits has no effect on the localization of either polymorphonuclear leucocytes or trypan blue in areas of injury.¹ Furthermore, this anticoagulant does not affect the development of an acute inflammatory reaction in the skin of the rabbit. Heparin, likewise, does not inhibit the phagocytosis of particles of India ink by the cells of the reticulo-endothelial system.² To these observations now may be added the observation that heparin has no effect on either the agglutination of human red blood cells by heterologous serum or the agglutination of typhoid bacilli by immune sera. Heparin also has no fibrinolytic effect on clots of normal and parasitized chicken blood.

There apparently is no evidence from the study of the pathologic lesions occurring in the monkeys infected with *P. knowlesi*, and too, from the study of the effects of heparin on the parasitemia in chicks to support the opinion that fibrin or a fibrinlike substance coats parasitized erythrocytes to produce clumping and to inhibit phagocytosis. Immunologic reactions characterized by agglutination are well known in which fibrin does not occur. Holmes⁴ has recently reviewed this problem and discussed the phenomena of agglutination and precipitation in relation to surface tension.

Robert Fahraeus¹⁵ in 1929 reported in man the presence of intravascular aggregation of erythrocytes identical to those described by Kinsely and associates.⁶ In this paper Fahraeus reviewed the phenomena of rouleau formation, intravascular aggregation of erythrocytes as observed in disease and the mechanism of the cellular changes as observed in both the normal and in certain pathological conditions.

Many of the normal chicks died during the period in which heparin was injected. Hemorrhages were present in the tissues inoculated and also in the serous cavities. Chicks infected with malaria and given heparin also died during the time of the experiment. They showed hemorrhages similar to those in the control group. This bleeding into the serous cavities of the chick is interesting in view of previous observations upon capillary permeability in rabbits given heparin.¹ In studies in the rabbit it was believed that "heparin has no effect on capillary permeability." It is now obvious from our present observations in the chick that both the type of animal and the quantity of heparin must be considered in studying the effect of heparin on capillary permeability. It would appear from the results of many experimental observations that capillary permeability may be affected by heparin. The use of heparin in clinical cases of acute malaria is contraindicated since hemorrhages do occur in the viscera in this disease.¹⁶ The presence of this anticoagulant may facilitate the escape of red blood cells through the capillary walls and thereby produce larger hemorrhages in the parenchymatous tissues.

CONCLUSION

There is no evidence from this study to support the opinion that heparin inhibits the process of phagocytosis of malarial parasites. The parasitemia is

less in chicks given heparin immediately following the injection of the parasites than it is in the controls.

Heparin apparently has no effect upon either the agglutination of human red blood cells by heterologous serum or the agglutination of typhoid bacilli by immune sera.

Heparin has no effect apparently, on previously formed clots of normal and parasitized chicken blood.

The role of heparin in other of the fundamental biological processes is briefly referred to.

REFERENCES

1. Rigdon, R. H., and Wilson, Harwell: Capillary Permeability and Inflammation in Rabbits Given Heparin, *Arch. Surg.* 43: 64, 1941.
2. Rigdon, R. H., and Schrantz, F. S.: Effect of Heparin on Phagocytosis by the Cells of the Reticulo-Endothelial System, *Ann. Surg.* 116: 122, 1942.
3. Von Jansco, N.: Pharmakologische Beeinflussung des Reticuloendothels, *Klin. Wchnschr.* 10: 537, 1931.
4. Capell, D. F.: Intravital and Supravital Staining, *J. Path. and Bact.* 32: 629, 1929.
5. Wright, A. E., and Douglas, S. R.: An Experimental Investigation of the Role of the Blood Fluids in Connection With Phagocytosis, *Proc. Roy. Soc., London S. B.* 72: 364, 1903.
6. Kinsely, M. H., Stratman-Thomas, W. K., and Elliott, Theodore, S.: A Consideration of the Circulatory Phenomena Observed in Malaria, *Anat. Record* 79: 90, 1941.
7. Kite, G. L., and Wherry, W. B.: The Mechanism of Phagocytosis, *J. Infect. Dis.* 16: 109, 1915.
8. Rabinovitch, Jacob: The Cause of Death Following Intravenous Injections of Ox and Dog Serum in Rabbits, *Arch. Path.* 7: 615, 1929.
9. Rigdon, R. H., and Stratman-Thomas, W. K.: A Study of the Pathological Lesions in P. Knowlesi Infection in M. Rhesus Monkeys, *Am. J. Trop. Med.* 22: 329, 1942.
10. Quick, Armand J.: The Hemorrhagic Diseases and the Physiology of Hemostasis, Charles C Thomas, Springfield, Ill., 1942.
11. Schuffner, W. A. P., and Esseveld, H.: Observations on the Malignancy of Malignant Malaria, *Geneesk. tijdschr. v. Nederl. Indie* 47: 3038, 1936. (abst.) *Trop. Dis. Bull.* 34: 383, 1937.
12. Cropper, J.: Agglutination of Parasitized Red Blood Cells, *Lancet* 2: 16, 1903.
13. Best, C. H., Cowan, C., and McLean, L.: Heparin and the Formation of White Thrombi, *J. Physiol.* 92: 20, 1938.
14. Holmes, Lida F.: The Effect of Surface Tension Depressants on Certain Serological Systems, *Yale J. Biol. & Med.* 14: 155, 1942.
15. Fahraeus, Robin: The Suspension Stability of the Blood, *Physiol. Rev.* 9: 241, 1929.
16. Rigdon, R. H.: A Consideration of the Mechanism of Death in Acute Plasmodium Falciparum Infection; Report of a Case, *Am. J. Hyg.* 36: 269, 1942.

EFFECT OF GONADS AND ADRENALS ON THE ABSORPTION OF SUBCUTANEOUS SESAME OIL

CHARLES E. TOBIN, PH.D., ROCHESTER, N. Y.

MANY plant oils, used as solvents for hormones, are considered to be without effect on the injected subject. Evidence is being accumulated, however, to show that such oils are not bland when injected into certain animals. The oil used may either have various effects on the animal, or hormones may influence absorption of the oil.¹⁻¹³

METHODS

To test the effect of hormones from the animal's own gonads and adrenals on the rate of absorption of subcutaneous sesame oil, the following experiments were performed on adult albino rats in groups of ten animals each. The rats were kept in a thermostatically controlled room and given tap water and a commercial diet (Wayne Dog Blox) ad libitum. One cubic centimeter of sesame oil was injected subcutaneously, as 0.5 c.c. doses, at two sites over the left flank into normal, adrenalectomized, or adrenalectomized-gonadectomized rats (at the time of operation), and gonadectomized animals (2 weeks after operation). All animals were killed fourteen days after injection, except the pregnant ones, which were operated upon and injected on the tenth day of pregnancy, and killed twenty-one days after delivery.

The procedure for recovering the oil was similar to that reported by Turner and Mulliken.^{2,3} The initial weight of 1 c.c. of sesame oil was 915 ± 4.5 mg.* Bruce and Tobin⁴ have reported the composition and other properties of this oil from a similar source. A comparable area of skin and subcutaneous tissue taken from the right flank of each animal was extracted similarly to indicate the amount of ether-soluble matter, other than injected oil, present in the recovered oil. Corrections are made in the data for the weight of this matter.

RESULTS

The amount of oil absorbed by normal animals is proportional to the length of time the oil remained in the animals: a group of normal males killed 30 minutes, and another group killed 24 hours after injection, absorbed 299 ± 58 mg. and 414 ± 99 mg. of oil, respectively. Increasing amounts of oil were absorbed with longer experimental periods: males killed 12 days after injection absorbed 473 ± 187 mg. of oil. Both control and experimental animals were studied, for similar periods, in order to control the time element on the rate of absorption of the subcutaneous sesame oil.

The significance of the differences in the amount of oil absorbed by the various groups was tested by Fisher's "t" method. Only those differences

From the Department of Anatomy, The University of Rochester School of Medicine and Dentistry.

Received for publication, April 2, 1944.

*The accuracy of the recovery method employed is indicated by the weight of oil run through the above procedure which varied only by ± 9.2 mg. from the initial weight of the oil.

whose "t" values were 2.10 ($P = 0.05$) or greater were considered significant and are discussed below. The amount of oil absorbed by the normal and operated animals is shown in Table I.

Effect of Gonads.—Normal females absorbed the greatest quantity of oil, the castrate males and normal males were next in descending order, whereas spayed females absorbed the smallest amount of oil in this normal or gonadectomized group. Significant differences were found in the amounts of oil absorbed by the spayed females compared to normal females ("t" value 2.9),

TABLE I
ABSORPTION OF SESAME OIL FROM THE SUBCUTANEOUS TISSUE OF RATS

Oil absorbed†	*Normal ♀	*Castrate ♂	*Normal ♂	*Adrenalectomized ♂	*Adrenalectomized ♀	*Spayed ♀	*Adrenalectomized castrate ♂	*Adrenalectomized spayed ♀	†Virgin ♀	†Pregnant adrenalectomized ♀	†Pregnant normal ♀
Per cent	55	52	51	49	42	31	31	25	72	63	51
Avg. mg.	512	479	473	451	386	288	284	233	605	578	467
σ mg.	197	203	187	162	207	143	142	123	124	156	186

*Killed 14 days after injection.

†Killed 31 days after injection.

‡Based on initial weight of 1-c.c. oil, 915 ± 4.5 mg., injected.

$$\sigma = \sqrt{\frac{2d^2}{\pi r^2}}$$

castrate males (2.4), or normal males (2.5). However, no significant differences were found between the amount absorbed by normal females or by castrate or normal males. There was likewise no significant difference in the oil absorbed by castrate and normal males. The sesame oil injection had no effect on the reproductive physiology of the normal rats, and influenced in no way the atrophy of the accessory reproductive organs resulting from gonadectomy.

Effect of Adrenals.—Although bilateral adrenalectomy appeared to decrease the amount of oil absorbed by rats, this difference was not significant. There was a significant difference only when the amounts absorbed by adrenalectomized males were compared with those absorbed by spayed females (2.4).

Effect of Gonads and Adrenals.—Removal of both the gonads and the adrenals from rats reduced the amount of oil absorbed by such animals. This is shown by the differences in absorption of oil by adrenalectomized-castrate males and adrenalectomized-spayed females, respectively, compared with that of normal females (3.0 and 3.8), castrate males (2.5 and 3.3), normal males (2.6 and 3.4), and adrenalectomized males (2.5 and 3.4). The adrenalectomized-castrate males absorbed 31 per cent while the adrenalectomized-spayed females absorbed 25 per cent, indicating that the ovaries may influence slightly the utilization of sesame oil in adrenalectomized animals. However this latter difference was not significant.

Effect of Normal Pregnancy and Adrenalectomy During Pregnancy.—Since these experiments were of longer duration than the preceding ones (31

days), the amounts of oil absorbed cannot be compared directly with those from the nonpregnant groups. However, as compared with virgin females studied for a similar period, which absorbed 72 per cent of the oil, the adrenalectomized-pregnant females absorbed 63 per cent, and the normal-pregnant females utilized relatively less, 51 per cent. Only 50 per cent of the adrenalectomized-pregnant animals delivered; three of these lactated sufficiently to maintain 63 per cent of their litters until the twenty-first day. A significant difference was found between the amounts of oil absorbed by the virgin females and pregnant normal females (2.8); but no significant difference was found when the data from the pregnant adrenalectomized females were compared with that from the virgin females or normal pregnant rats.

CONCLUSIONS

The rate of absorption of sesame oil from rats is reduced by removal of the ovaries, removal of the adrenals in addition to the gonads, and by pregnancy. The sesame oil was injected subcutaneously in 1 c.c. amounts and recovered later as an ether-soluble extract of the injection sites.

REFERENCES

1. Zarrow, M.: Protective Action of Desoxycorticosterone Acetate and Progesterone in Adrenalectomized Mice Exposed to Low Temperatures, *Proc. Soc. Exper. Biol. & Med.* 50: 135, 1942.
2. Turner, J. C., and Mulliken, B.: Rate of Disappearance of Subcutaneous Vegetable Oil in Normal and Castrate Mice, *Proc. Soc. Exper. Biol. & Med.* 48: 598, 1941.
3. Turner, J. C., and Mulliken, B.: Effect of Androgen on Metabolism of Subcutaneous Corn-Oil, *Proc. Soc. Exper. Biol. & Med.* 49: 585, 1942.
4. Bruce, R. B., and Tobin, C. E.: The Effects of Sesame Oil and Fractions of Sesame Oil on Adrenalectomized and Other Experimental Rats, *Endocrinology* 27: 956, 1940.
5. Clausen, H. J.: The Atrophy of the Adrenal Cortex Following the Administration of Large Amounts of Progesterone, *Endocrinology* 27: 989, 1940.
6. Crafts, R. C.: Sesame Oil as a Vehicle for Fat Soluble Hormones, *Endocrinology* 31: 142, 1942.
7. Pollia, J. A.: The Effects of Lard Oil, Sesame Oil, Acacia, Retene and 1:2:5:6 Dibenzanthracene on Certain Organs and a Transplantable Rat Sarcoma in Animals of Pure Breed, *Radiology* 29: 683, 1937.
8. Spurr, C. L., and Kochakian, C. D.: The Effect of Androgen on the Survival of Adrenalectomized Rats, *Endocrinology* 25: 782, 1939.
9. Stein, K. F., and Allen E.: Attempts to Stimulate Proliferation of the Germinal Epithelium of the Ovary, *Anat. Rec.* 82: 1, 1942.
10. Tobin, C. E.: The Effect of Adrenalectomy on Pregnancy and Survival of Untreated and Sesame Oil Treated Rats, *Endocrinology* 28: 419, 1941.
11. Emery, F. E., Matthews, C. S., and Schwabe, E. L.: The Absorption of Stilbesterol and Theelin From Cysts of Sesame and Peanut Oils, *J. LAB. & CLIN. MED.* 27: 622, 1942.
12. Emery, F. E., and Matthews, C. S.: A Note on Cysts and Abscesses Induced in the Rat by Injections of Oils, *J. LAB. & CLIN. MED.* 28: 1795, 1943.
13. Brown, W. E., Wilder, V. M., and Schwartz, P.: A Study of Oils Used for Intramuscular Injections: A Study of the Physical, Chemical and Biologic Factors, *J. LAB. & CLIN. MED.* 29: 259, 1944.

CLINICAL CHEMISTRY

THE EFFECT OF BILE ACIDS ON THE BILIARY EXCRETION OF NEOARSPHENAMINE AND MAPHARSEN

J. H. ANNEGERS, M.S., F. E. SNAPP, M.S., A. C. IVY, M.D., AND
A. J. ATKINSON, M.D., CHICAGO, ILL.

IT HAS been reported that the administration of sodium dehydrocholate ameliorates the jaundice and anorexia of arsenical hepatitis even when the arsenical injections are continued.¹ It has also been reported that in rabbits when neoarsphenamine was injected with sodium dehydrocholate, less arsenic was recovered from the liver than when the drug was injected alone.^{1,2} It is well established that arsenic is excreted in the bile following injection of arsphenamines, but the rate of excretion has not been studied in permanent bile-fistula animals.

In view of the extensive use of neoarsphenamine and mapharsen and the occasional occurrence of a clinically evident hepatitis when they are used, it was considered worth while to investigate (a) the rationale of the claim that the administration of dehydrocholic acid has a favorable effect in arsenical therapy and (b) the effect of bile acids on the excretion of arsenic.

Dehydrocholic acid (oxidized cholic acid) may act to prevent the occurrence of an arsenical hepatitis in several ways. (a) The bile acid causes a hydrocholeresis³ which may augment the excretion of arsenic in the bile and decrease the exposure of the liver to arsenic. (b) Dehydrocholic acid increases the hepatic arterial blood flow⁴ which may, through the consequent increased oxygen supply, protect the liver by increasing its metabolism and its ability to detoxify harmful substances. (c) The bile acid may combine with the arsenical in such a way as to render it less hepatotoxic. (d) Since bile salts injected intravenously increase the permeability of the capillaries to certain compounds,⁵ the simultaneous injection of dehydrocholic acid and the arsenical may cause more of the latter to pass into the general tissues than otherwise, and thus decrease the exposure of the liver to the arsenical. This would expose other tissues to more of the arsenical, which may or may not be a desirable action depending upon whether the toxic effect on other tissues or the treponemicidal action of the drug was more markedly increased.

This investigation has been confined to the study of the effect of two types of bile acids on the excretion of arsenic in the bile.

From the Department of Physiology, Northwestern University Medical School, Chicago.
Received for publication, January 31, 1911.
This study was assisted by a grant from the Eli Lilly Company.

GENERAL METHODS

Two types of bile acids were used. One was conjugated, unoxidized bile acids represented by sodium glycocholate and taurocholate (Bilron, Lilly). The other was an unconjugated, oxidized bile acid represented by sodium dehydrocholate (Decholin, Riedel de Haen, and Ketchol, Searle). The former preparation, which we shall hereafter refer to as "ox-bile" acid, because it was made from cattle bile, causes a moderate choleresis but does not increase the hepatic arterial blood flow. The latter preparation or sodium dehydrocholate causes a hydrocholeresis and increases the hepatic arterial blood flow.

Biliary fistula dogs with a duodenal fistula for the return of bile into the intestine were used⁶ for the experiments extending over a week or more. Acute bile fistula dogs under nembutal anesthesia were used for the tests lasting only three or four hours.

The bile was analyzed for arsenic by the method of Cassil and Wichman.⁷ This method was reported by them to be accurate for amounts of arsenic varying from 5 to 500 micrograms in the sample analyzed. We obtained the best duplicate checks when the sample contained less than 300 micrograms.

The neoarsphenamine injections consisted of one dose of 300 mg. which contained 60 mg. of arsenic. The mapharsen injections consisted of one dose of 60 mg. which contained approximately 17.4 mg. of arsenic. These doses range from 2 to 4 times the *single* weekly human therapeutic dose on the basis of body weight. However, when mapharsen is used in the intensive treatment of syphilis, from 1.0 to 1.2 Gm. is given in a five-day period.^{8,9} This dose averages 240 mg. daily, and amounts to four times the dose used in this study.

The dogs invariably vomited upon receiving mapharsen. For this reason the drug was always given at least ten hours after a meal. This was done because the amount of cholic acid formed depends on the protein absorbed and metabolized, and in order to maintain the animals under controlled conditions, food intake must be constant.

Arsenical injections were not repeated in the same animal more often than once a week, and the sequence of the tests was rotated so that the animals did not all receive the drugs and the drugs plus bile acids in the same order. The volume output of bile was recorded during the tests, but is not included in the data. The average volume output is not changed significantly by the arsenical injections.¹⁰

RESULTS

Neoarsphenamine

The rate of disappearance of arsenic from the bile after a single intravenous injection of neoarsphenamine: In this series of tests the animals were fed the standard diet every twelve hours; bile was not returned to the intestine, nor were bile acids given orally. Three hundred milligrams of neoarsphenamine were injected and the twenty-four-hour bile output was analyzed for arsenic.

The results are shown in Table I. Most of the arsenic was excreted during the first twenty-four hours after the injection and relatively small amounts were excreted after seventy-two hours. Traces of arsenic were present in the

TABLE I

SHOWING THE DAILY BILIARY EXCRETION OF ARSENIC FOLLOWING INJECTION OF 300 MG. NEOARSPHENAMINE CONTAINING 60 MG. ARSENIC. NO BILE OR BILE ACIDS WERE GIVEN*

MG. BILIARY ARSENIC EACH DAY AFTER THE DOSE									% OF DOSE RECOVERED IN BILE
DOG	1 DAY	2 DAYS	3 DAYS	4 DAYS	5 DAYS	6 DAYS	7 DAYS	TOTAL	
C4	18.83	1.88	.82	.73	.53	.58	.75	18.11	31
C4	17.09	15.72	2.81	.66	.62	.46	.29	37.55	63
C7	16.08	4.42	2.10	.49	.93	.63	Trace	24.65	41
D9	19.33	2.22	2.22	.92	Trace	Trace	Trace	24.69	41

*These tests show that most of the arsenic excretion occurs in 72 hours after giving the drug. In subsequent tables arsenic excretion is given for 24- and 72-hour periods following the dose of neoarsphenamine.

bile seven days after the injection. From 27 to 32 per cent of the injected arsenic was recovered in the bile in twenty-four hours, and from 31 to 63 per cent of the injection was excreted in the bile in seven days.

The effect of "ox-bile" acids and dehydrocholic acid on the excretion of arsenic after a single injection of neoarsphenamine: In this series of tests a group of bile fistula dogs were fed the standard diet every twelve hours until the volume and cholic acid output were constant. Three hundred milligrams of neoarsphenamine were injected intravenously. The bile was collected every twenty-four hours for three days and analyzed for arsenic. A seventy-two-hour period was chosen because the bile was relatively free of arsenic after that time.

Twelve dogs (Table II) excreted an average of 24 mg. of arsenic in seventy-two hours, or 40 per cent of the amount injected. This was the control series. When 1.5 Gm. of "ox-bile" acid was fed every twelve hours for three days before and after giving neoarsphenamine, an average of 26.9 mg., or 44 per cent of the arsenic was recovered in the bile. When 1.5 Gm. of dehydrocholic

TABLE II

SHOWING EFFECT OF "OX-BILE ACIDS" AND OF DEHYDROCHOLIC ACID ON EXCRETION OF ARSENIC IN BILE FOR A PERIOD OF 72 HOURS AFTER INTRAVENOUS INJECTION OF 300 MG. NEOARSPHENAMINE CONTAINING 60 MG. ARSENIC

MG. ARSENIC EXCRETED IN 72 HOURS								
CONTROL. NO BILE ACID 3 CM. "OX-BILE ACIDS"						3 CM. DEHYDROCHOLIC ACID		
DOC	MG.	% OF DOSE	MG.	% OF DOSE	% CHANGE	MG.	% OF DOSE	% CHANGE
C4	35.5	59.4				20.8	33.6	-42
C7	23.6	39.3				14.8	24.0	-38
D9	23.8	39.6	43.9	73.0	+ 85			
F3	21.2	35.3	22.7	37.8	+ 7	24.1	40.0	+14
F6	18.9	31.4	14.6	24.4	- 23	16.7	27.8	-12
F7	25.0	41.8	26.8	44.6	+ 7			
F8	35.7	59.6	15.6	26.0	- 57	29.4	49.0	-18
F9	17.6	29.4	36.1	60.0	+104			
F10	20.2	33.6	19.7	33.0	- 2	10.4	17.4	-50
G1	23.5	39.2	35.1	58.5	+ 50	28.3	47.2	+21
G5	25.3	42.2	30.1	50.0	+ 19	21.8	36.4	-14
						25.9	43.1	+ 2
G6	17.7	29.6	21.3	36.0	+ 20	14.7	24.4	-17
						19.2	32.0	+ 8
Average	24	40.1	26.9	44.3	+ 15	20.5	34.1	-16
					+ 12			-15

$$\frac{26.9 - 24.0}{24} = +12\%$$

$$\frac{24.0 - 20.5}{24} = -15\%$$

acid was similarly fed, an average of 20.5 mg., or 34 per cent of the dose, was eliminated in the bile in seventy-two hours.

Thus, the administration of "ox-bile" apparently increased the excretion of arsenic by an average of 15 per cent as compared with the control series, and the feeding of dehydrocholic acid decreased arsenic excretion by an average of 16 per cent. A statistical analysis of the data, however, revealed that the average increase of 15 per cent and the average decrease of 16 per cent as compared with the control were not significant. Nevertheless, the difference between the arsenic excretion when "ox-bile" was fed and when dehydrocholic acid was fed was sufficiently statistically significant to indicate that it was probably not due to chance variation. This difference suggests only that dehydrocholic acid tends to retard the biliary excretion of arsenic as compared to the excretion when "ox-bile" acids are given.

Comment.—Sodium dehydrocholate and "ox-bile" acids increase biliary volume output on the average by 100 per cent and 35 per cent,³ respectively. Since, when neoarsphenamine was given, significantly more arsenic was not excreted in the bile when bile acid was given than when none was given, *choleresis, per se, does not augment the biliary excretion of arsenic* administered in the form of neoarsphenamine.

The differences in the excretion of arsenic in the control series and the series given "ox-bile" acids and dehydrocholic acid cannot be attributed to previous arsenic administration. In one-third of the animals the control tests were performed last; in one-third the "ox-bile" acid was given last, and in one-third the dehydrocholic acid was given last. Further, at least one week elapsed between arsenical injections which is sufficient time to clear the bile of measureable amounts of arsenic.

To make certain that the presence of choleresis was not associated with a greater excretion of arsenic during the first 24 hours, the biliary arsenic for the first twenty-four hours of the tests was tabulated. The data shown in Table III show that arsenic excretion in the bile was not increased the first twenty-four hours in the series given either bile acid preparation.

The effect of injecting neoarsphenamine with sodium dehydrocholate on the excretion of arsenic in the bile: Appel and Jankelson² found that when a mixture of sodium dehydrocholate and neoarsphenamine was injected into rabbits, less arsenic was found in the liver after two to twenty-four hours than when neoarsphenamine was given alone. This observation was interpreted as indicating that the dehydrocholate caused more arsenic to be excreted in the bile. Our results on the dog do not support such a conclusion. It is possible, however, that different results might be obtained when the bile salt is mixed with the neoarsphenamine in the same syringe rather than giving the dehydrocholate orally. The following experiments were performed to examine this possibility.

Chronic or permanent biliary fistula dogs were so prepared that their collecting tube system contained about 5 c.c. of bile. Such animals cannot be used satisfactorily for tests lasting only an hour or so during which the output of bile may be less than 5 c.c. Therefore, "acute" or temporary bile fistula

TABLE III

SHOWING THE EFFECT OF "OX-BILE ACIDS" AND OF DEHYDROCHOLIC ACID ON EXCRETION OF ARSENIC IN BILE FOR A PERIOD OF 24 HOURS AFTER INTRAVENOUS INJECTION OF 300 MG. NEOARSPHENAMINE CONTAINING 60 MG. ARSENIC

DOG	MG. ARSENIC EXCRETED IN 24 HOURS							
	CONTROL: NO BILE ACID		1.5 GM "OX-BILE" ACIDS			1.5 GM. DEHYDROCHOLIC ACID		
	MG.	% OF DOSE	MG.	% OF DOSE	% CHANGE	MG.	% OF DOSE	% CHANGE
C4	17.09	28.5				12.39	23.0	-33
C7	16.08	26.8				11.65	19.4	-28
D9	19.33	32.2	29.98	50.0	+ 55			
F3	16.77	28.0	16.45	27.4	- 2	16.50	27.5	- 2
F6	11.37	19.0	9.15	15.3	- 18	10.10	16.8	-11
F7	17.58	29.3	19.65	32.8	+ 12			
F8	25.80	43.0	9.63	16.0	- 65	17.50	29.2	-32
F9	11.80	19.7	26.85	45.0	+128			
F10	14.50	24.2	13.85	23.0	- 3	5.64	9.4	-63
G1	15.95	26.6	25.20	42.0	+ 58	17.90	29.8	+12
G5	17.53	29.3	22.02	36.6	+ 25	16.78	28.0	- 4
						15.78	26.3	-10
G6	11.22	18.7	13.80	23.2	+ 23	12.10	20.2	+ 8
						10.01	16.7	-10
Average	16.25	27.1	18.66	31.1	+ 21	13.30	22.4	-16
					+ 14			-18

animals were prepared under nembutal anesthesia with the cystic duct clamped and a short collecting tube placed in the common bile duct.

The control series of dogs received 300 mg. of neoarsphenamine alone intravenously. A second series were given an injection of mixture of 300 mg. neoarsphenamine and 5 c.c. of 20 per cent sodium dehydrocholate. To this series additional injections of 5 c.c. of sodium dehydrocholate were given hourly. A third series of dogs received a single injection of dehydrocholate to induce a brisk choleresis, and ten minutes later were given 300 mg. of neoarsphenamine.

TABLE IV

SHOWING THE EFFECT OF INTRAVENOUS SODIUM DEHYDROCHOLATE ON THE BILIARY EXCRETION OF ARSENIC DURING THREE HOURS AFTER AN INJECTION OF 300 MG. OF NEOARSPHENAMINE CONTAINING 60 MG. OF ARSENIC*

DOG	WT. KG.	ONE HOUR		TWO HOURS		THREE HOURS		TOTAL			PROCEDURE
		VOL. IN C.C.	MG. AS.	VOL. IN C.C.	MG. AS.	VOL. IN C.C.	MG. AS.	VOL. IN C.C.	MG. AS.	% REC.	
1	12.0	1.4	0.16	5.6	1.75	5.6	1.12	12.6	3.12	5.0	Series I. Controls; no bile acid
3	11.2	6.6	1.57	6.8	1.70	5.8	1.46	19.2	4.74	7.8	
4	9.5	4.8	1.54	5.4	3.20	6.8	3.37	17.1	8.13	13.5	
Ave.		4.3	1.10	5.9	2.20	6.1	2.01	16.3	5.33	8.8	
2	11.0	14.5	Trace	10.6	0.24	19.2	0.56	44.3	0.60	--	Series II. Mixture & 5 c.c. 20% sod. dehy- drocholate each hour
5	8.4	15.5	0.23	14.2	0.55	12.5	0.23	32.2	1.02	--	
Ave.		15.0	0.12	12.4	0.40	15.9	0.30	38.3	0.81		
6	9.7	25.5	2.70	22.5	6.09	4.5	0.90	52.5	9.69	16.0	Series III. One injection sod. dehydro- cholate 10 minutes before neo. injection
7	8.0	23.5	0.22	11.2	0.94	8.5	0.86	43.2	2.02	3.3	
8	6.6	14.2	0.60	11.0	1.15	8.6	0.83	33.8	2.58	4.3	
Ave.		21.1	1.17	14.9	2.73	7.2	0.86	43.2	4.76	7.9	

*Additional experiments by De Hoog and Gutmann in this laboratory confirm these findings.

The results are shown in Table IV. The choleresis produced by dehydrocholic acid did not increase the excretion of arsenic. This confirms the foregoing results from chronic biliary fistula dogs. The *simultaneous injection of sodium dehydrocholate and neoarsphenamine resulted in a marked decrease in the biliary excretion of arsenic*. These results indicate that the decreased retention of arsenic in the liver of rabbits when dehydrocholic acid was administered with neoarsphenamine observed by Appel and Jankelson was probably not due to increased excretion of arsenic in the bile. More likely, increased amounts of the arsenical passed into other tissues and this decreased the quantity of arsenic entering the liver.

Mapharsen

The effect of dehydrocholic acid on the excretion of arsenic in the bile after an intravenous injection of mapharsen: Table V presents the data from eighteen tests on ten dogs which were performed similarly to the experiments with neoarsphenamine. Sixty milligrams of mapharsen, containing 17.4 mg. of arsenic, were given to one series of dogs without feeding bile acids. To another series, 1.5 Gm. dehydrocholic acid was fed every 12 hours for 3 days before and after injecting the arsenical. Since it was found in confirmation of the work of Eagle and Hogan¹⁹ on rabbits that the excretion of arsenic in the bile after the administration of mapharsen was practically complete in forty-eight hours, Table V shows the biliary arsenic for this two-day period.

TABLE V

SHOWING EXCRETION OF MAPHARSEN IN BILE FOR A PERIOD OF 48 HOURS FOLLOWING INJECTION OF 60 MG. OF THE DRUG CONTAINING 17.4 MG. ARSENIC. EIGHT TESTS SHOW THE RESULTS WHEN NO BILE ACIDS WERE GIVEN. TEN TESTS SHOW EFFECT OF DEHYDROCHOLIC ACID, 1.5 GM. EVERY 12 HOURS ON EXCRETION OF ARSENIC

DOG	NO BILE ACID			DOG	DEHYDROCHOLIC ACID		
	MG. ARSENIC IN 48 HOURS	PER CENT OF DOSE	% CHANGE		MG. ARSENIC IN 48 HOURS	PER CENT OF DOSE	% CHANGE
F3	7.90	45.4		G5	7.06	41	
F8	8.92	51.2		G5	4.92	28	
G1	9.32	53.5		G6	10.40	60	
G1	9.74	56.0		G6	9.16	53	
G2	6.87	39.5		G8	6.21	36	
G3	10.03	57.5		G8	6.01	34	
G5	5.47	31.4		G9	4.94	28	
G6	5.84	33.6		G9	5.72	33	
				G10	5.07	29	
				G10	5.26	30	
Ave.	8.02	46.0	-		6.47	37.2	-19

The results in Table V show that the administration of dehydrocholic acid was not associated with an increase in arsenic excretion, though the bile acid had a marked choleric action. In fact, the excretion of arsenic was 19 per cent less on the average than that observed in the control series when no dehydrocholic acid was given. Although this difference is not statistically significant, the tendency for dehydrocholic acid to decrease excretion of mapharsen-arsenic is similar to that observed for neoarsphenamine-arsenic.

It is worthy of noting that the percentage recovery of mapharsen-arsenic at forty-eight hours (Table V) is approximately the same as that of neo-

arsphenamine-arsenic at seventy-two hours (Table II). This shows that mapharsen-arsenic is more rapidly excreted in the bile than neoarsphenamine-arsenic.

The total excretion of neoarsphenamine arsenic: Several reports indicate that arsenic is excreted in the feces and to a lesser extent in the urine.¹¹⁻¹³

In one biliary fistula dog the amount of arsenic excreted in the bile and in the urine was determined after injecting 300 mg. of neoarsphenamine. In this experiment 65 per cent of the arsenic was recovered in the bile and 30 per cent in the urine after seventy-two hours.

Is the arsenic excreted in the bile reabsorbed in the intestine? Though it is known that organic arsenicals are poorly absorbed from the intestine, the form in which arsphenamines appear in the bile has not been determined. The bile from two dogs was collected for 24 hours following injections of 300 mg. neoarsphenamine. This bile contained 25 and 30 mg. of arsenic, respectively, and was returned to the duodenum of two other bile fistula dogs which had never before received arsenic. No arsenic was recovered from the bile of the second pair of dogs. Thus the biliary arsenic after injecting neoarsphenamine was not absorbed from the intestine.

Does arsenic accumulate in the bile after repeated weekly injections of neoarsphenamine or mapharsen? When 300 mg. of neoarsphenamine or 60 mg. of mapharsen was injected weekly for 5 to 8 weeks, the bile was repeatedly free of arsenic after seven days and no evidence of delayed excretion or accumulation of arsenic in the bile was observed.

DISCUSSION

The excretion of arsenicals by the liver of the dog: The results of this study confirm by a more direct method the conclusion of other investigators¹⁴⁻¹⁷ that the liver is the predominant organ concerned in the excretion of arsenicals, and that the excreted arsenic does not undergo an enterohepatic circulation or absorption from the intestine. Within two or three days after injection, from 30 to 60 per cent, or an average of about 40 per cent, of the arsenic administered as neoarsphenamine or mapharsen in well-tolerated doses is excreted in the bile. It appears that mapharsen-arsenic is more rapidly excreted than neoarsphenamine arsenic. This may not be true, since 60 mg. of arsenic was given as neoarsphenamine while 17.4 mg. of arsenic was available for excretion after injecting mapharsen. It is of interest to observe that mapharsen may be more readily excreted, since it has been suggested that neoarsphenamine is converted to arsenoxide, or mapharsen, before it becomes actively treponemicidal in the body.

The process concerned in the excretion of mapharsen and neoarsphenamine is not one of simple diffusion. This is shown by the relatively slow excretion and by the failure of a choleresis to increase significantly the rate of arsenic excretion. These arsenical compounds, which are predominately removed from the blood by the liver, cannot be flushed out of the liver by producing a choleresis with bile acids.

It is evident that an increase in hepatic arterial blood flow does not facilitate the excretion of arsenicals. Sodium dehydrocholate, given intravenously or by mouth, increases hepatic arterial flow but does not increase the excretion of the arsenicals.

Do bile acids differ in their effect on the excretion of arsenical drugs? It has been clearly demonstrated that the liver handles conjugated unoxidized (glycocholic and taurocholic acids—Bilron, Lilly) and oxidized unconjugated (dehydrocholic acid—Ketochole and Dechole) bile acids differently.³ When "ox-bile" acids are fed at least 90 per cent of them can be recovered in the bile as cholic acid. When dehydrocholic acid is fed or injected, only about 30 per cent can be recovered in the bile; the fate of the remainder is unknown. Probably, the oxidized bile acid is chemically changed and some of it may reach the peripheral circulation.

Our data show that glycocholic and taurocholic acids tend to increase and dehydrocholic acid tends to decrease the excretion of arsphenamine-arsenic in the bile.

One cannot conclude from the evidence in this paper that one type of bile acid would be more beneficial in protecting the liver from arsenical hepatitis than the other type of bile acid. The apparent decreased elimination of arsenic in the bile when dehydrocholic acid is given may not be due to retention of arsenic in the liver. On the contrary, the bile acid may pass into the peripheral circulation and increase capillary permeability to the arsenical and increase the amount of the arsenical in the general systemic tissues. If this is true, dehydrocholic acid would decrease the exposure of the liver to arsenic.

The results of giving dehydrocholic acid intravenously mixed with neoarsphenamine support the possibility that the bile acid allows the arsenical to pass more readily from the blood stream into tissues other than the liver only. Arsenic excretion in the bile was markedly decreased when the bile acid and neoarsphenamine were mixed and injected. When dehydrocholic acid was injected ten minutes prior to the arsenical injection, arsenic excretion was not decreased. Thus, when dehydrocholic acid is present in the circulation, simultaneously with neoarsphenamine, apparently less of the arsenical reaches the liver. Dehydrocholic acid is largely removed from the circulation in ten minutes after a single injection, and acts primarily upon the liver producing a choleresis, its effect on capillary permeability being transitory. This explanation is supported by the observations of Berman, Snapp and Ivy⁵ on the effect of intravenously administered bile salts on the elimination of intravenously injected bilirubin. Bile salts injected with bilirubin decreased the amount of bilirubin excreted in the bile and increased the amount which entered the tissues, to such an extent that visible icterus occurred.

The effect of mixing sodium dehydrocholate with mapharsen on the excretion of arsenic in the bile was not studied because when the two are mixed a precipitate forms.

The "natural bile salts," sodium glyco- and taurocholate, were not used for intravenous injection in this study because they are more toxic¹⁸ when given parenterally than sodium dehydrocholate.

Is it rational to give bile acids in an attempt to avoid arsenical hepatitis? If the observations on rabbits and on dogs hold true for man, the decreased amount of arsenic found in the liver and the amelioration of the toxic reactions observed by Appel and Jankelson^{1, 2} when neoarsphenamine was administered with sodium dehydrocholate, is not due to greater excretion of the arsenical in

the bile. It is more likely due either to a greater dilution of the arsenical in the general body tissues, sparing the liver somewhat, or to a combination of the dehydrocholate with the arsenical so it becomes less toxic. The question will have to be answered by direct experiments in which the toxicity of the arsenical is compared when injected with and without dehydrocholic acid.

The accumulation of arsenic in the body in intensive syphilotherapy. It has been shown that nearly complete excretion of arsenic from a single injection of 60 mg. of mapharsen occurs within forty-eight hours in biliary fistula dogs, while neoarsphenamine-arsenic is largely excreted in seventy-two hours. In the five-day treatment of syphilis when the massive arsenotherapy method is used, mapharsen is given to the extent of 240 mg. in 24 hours for five days.⁸⁻⁹ Thus, an injection of 60 mg. is repeated approximately every six hours. If arsenic is excreted in the human being as in the dog, considerable accumulation of arsenic will occur in five days, most of which would be in the liver.

According to observations made in this laboratory, the L. D. 50 of mapharsen or the dose required to kill 50 per cent of the animals is the same for dogs as for rabbits, namely, 13 mg. per kilo. In the rabbit, the results of Eagle and Hogan¹⁰ indicate that a sublethal dose of mapharsen may be injected weekly without accumulative effect. Our results on the dog confirm their observation on the rabbit.

SUMMARY AND CONCLUSIONS

1. The excretion of neoarsphenamine and mapharsen-arsenic in the bile of dogs with a permanent bile fistula has been studied under standard conditions with and without the oral administration of a mixture of sodium glycocholate and taurocholate (ox-bile salts) and sodium dehydrocholate. The dose of neoarsphenamine was 300 mg. or 60 mg. of arsenic, and the dose of mapharsen was 60 mg., or 17.4 mg. of arsenic. The dose of arsenic was not given oftener than once a week and the animals weighed from 8 to 12, or an average of 10 kilograms.

2. In the case of neoarsphenamine, an average of 40 per cent of the arsenic administered was recovered in the bile in seventy-two hours. In the case of mapharsen, an average of 40 per cent of the arsenic administered was recovered in the bile in forty-eight hours. In both cases most of the arsenic is excreted in the bile during the first twenty-four-hour period and only traces are present in the bile after forty-eight to seventy-two hours. The results indicated that neoarsphenamine-arsenic is excreted more slowly by the liver than mapharsen-arsenic. It was shown that the arsenic excreted in the bile is not absorbed from the intestine.

3. If neoarsphenamine or mapharsen-arsenic is excreted by the human liver as by the canine liver, it is reasonable to assume that an appreciable accumulation of arsenic occurs in the liver when, for example, 240 mg. of mapharsen is administered daily for five days. However, in confirmation of Eagle and Hogan¹⁰ and Magnuson and Raulston¹⁴ who used rabbits, a sublethal dose may be given weekly without accumulation of arsenic in the liver.

4. The production of a choleresis *per se* in the dog does not augment the rate of excretion of neoarsphenamine or mapharsen-arsenic in the bile, because the oral administration of sodium dehydrocholate, which causes a brisk choleresis,

tends to decrease the elimination of the arsenic, and sodium glycocholate and taurocholate, which cause only a moderate choleresis, tends to increase the elimination of the arsenic in the bile.

5. The simultaneous intravenous administration of sodium dehydrocholate with neoarsphenamine decreases the rate of excretion of the arsenic in the bile, though a choleresis occurs. This result is probably due to the increased passage of the arsenical into the general body tissues secondary to the effect of the bile salts on capillary permeability. If the administration of dehydrocholic acid diminishes the hepatotoxic action of neoarsphenamine and mapharsen,^{1,2} it does not do so by increasing their excretion in the bile.

6. The results of this investigation do not provide a clear and substantial rationale for the administration of bile acids with arsenicals.

REFERENCES

1. Appel, B.: Sodium Dehydrocholate in Arsphenamine Poisoning, *Arch. Dermat. & Syph.* 27: 401, 1933.
2. Appel, B., and Jankelson, I. R.: Treatment of Arsenical Hepatitis With Sodium Dehydrocholate, *Arch. Dermat. & Syph.* 32: 422, 1935.
3. Berman, A. L., Snapp, F. E., Ivy, A. C., Atkinson, A. J., and Hough, V. S.: The Effect of Various Bile Acids on the Volume and Certain Constituents of Bile, *Am. J. Digest. Dis.* 7: 333, 1940.
4. Grodins, F. S., Osborne, Ivy, A. C., and Goldman, L.: The Effect of Bile Acids on Hepatic Blood Flow, *Am. J. Physiol.* 132: 375, 1941.
5. Berman, A. L., Snapp, F. E., and Ivy, A. C.: The Effect of Choleresis on the Rate of Excretion of Intravenously Injected Bilirubin, *Am. J. Physiol.* 132: 176, 1941.
6. Kocour, E., and Ivy, A. C.: The Effect of Certain Foods on Bile Volume Output Recorded by a Quantitative Method, *Am. J. Physiol.* 122: 325, 1938.
7. Cassil, C. C., and Wichman, H. J.: The Determination of Arsenic, *J. A. O. A. C.* 22: 436, 1939.
8. Leifer, W., Chargin, L., and Hyman, H. T.: Massive Dose Arsenotherapy of Early Syphilis, *J. A. M. A.* 117: 1154, 1941.
9. Sadusk, J. F., Craige, B., Brookens, N., Poole, A. K., and Strauss, M. J.: Observations on Massive Dose Arsenotherapy of Early Syphilis, *Yale J. Biol. & Med.* 14: 333, 1942.
10. Annegers, J. H., Drill, V. A., Habegger, J., Ivy, A. C., and Atkinson, A. J.: The Effect of Neoarsphenamine and Mapharsen on Cholic Acid Synthesis and the Use of Dehydrocholic Acid to Diminish the Hepatotoxic Action of Mapharsen, *Arch. Dermat. & Syph.*, In Press.
11. Frenkel-Heiden and Navassart, E.: The Fate of Salvarsan in the Human Body, *Ztschr. f. exper. Path. u. Therap.* 13: 531, 1931.
12. Bulmer, F. M. R.: Experimental Research on Destruction and Elimination of Organic Arsenic Compounds After Intravenous Administration, *J. Pharmacol. & Exper. Therap.* 21: 301, 1923.
13. Del Baere, L. J.: The Fate of Neosalvarsan in the Human Body After Intravenous Injection, *Ztschr. f. d. ges. exper. Med.* 48: 24, 1925.
14. Magnuson, H. J., and Raulston, B. O.: Concentration of Arsenic in Tissues and Excretion of Arsenic in Experimental Animals Following Intravenous Injection of Massive Doses of Mapharsen, *Arch. Int. Med.* 14: 2199, 1941; *Ven. Dis. Inform.* 22: 431, 1941.
15. Kuroda, T.: The Excretion of Arsenic in Bile, *Arch. f. exper. Path. u. Pharmacol.* 120: 330, 1927.
16. Kraft, R. M., Harris, S., Robinson, C. S., and Gilliland, H.: Quantitative Studies on Arsenic Distribution and Excretion After Intravenous Injection of Neoarsphenamine, *Am. J. Syph., Gonorr. & Ven. Dis.* 22: 215, 1938.
17. Voegtlin, C.: Arsphenamine, *Physiol. Rev.* 5: 63, 1925.
18. Grodins, F. S., Berman, A. L., and Ivy, A. C.: Observations on the Toxicity and Cholcretic Activity of Certain Bile Salts, *J. Lab. & Clin. Med.* 27: 181, 1941.
19. Eagle, H., and Hogan, R. B.: *Ven. Dis. Inform.* 24: 33, 1943.

LABORATORY METHODS

GENERAL

APPLICATION OF THE "INDUCTOGRAPH" TO THE REGISTRATION OF MOVEMENTS, PARTICULARLY OF BODY STRUCTURES SUCH AS THE PYLORIC SPHINCTER

D. A. BRODY, M.D., AND J. P. QUIGLEY, PH.D., CLEVELAND, OHIO

THE fact that an inductorium produces, respectively, more or less current as the secondary coil approaches or recedes from the primary coil has been extensively employed in biology (chiefly for the production of induced current to stimulate tissue). The same principle can be adapted to produce an "induction diagraph" or "inductograph" to be employed in the registration of changing positions of body structures, e.g., the movements of the heart or blood vessels, the various portions of the digestive tube, the bladder, ureters, uterus, skeletal muscles, etc. For such studies two suitable electromagnetic coils (a primary and a secondary coil) are fastened at opposite sides of the tissue whose movement is being studied and as the tissue contracts or relaxes, the coils will approach or recede, and correspondingly more or less current will be induced in the secondary coil. The inductograph is well suited for use in chronic experiments on unanesthetized subjects using imbedded coils or coils fastened to external surfaces of the body. Also, for special purposes it appears to offer advantages over apparatus commonly employed in acute experiments on anesthetized animals.

In this laboratory, the inductograph has been extensively employed for a year and a half in studying gastrointestinal movements. The "pyloric inductograph" has been used in 32 dogs for the chronic study of the pyloric sphincter movements under what appears to be rather physiologic conditions. In these animals, the two electromagnetic coils described below were placed with aseptic precautions so the coil cores and the sphincter diameter were in the same line. That is, the coils were sutured diametrically opposite to each other to the outer surface of the pyloric sphincter surface at the points of attachment of the lesser omentum and the greater omentum, respectively (Fig. 1). The lead wires of the coils were sutured along the lesser and greater curvature of the pyloric antrum and finally were brought to the outside through a stab wound. The coils and the lead wires were both covered with omental tissue. Coils examined after having been in place for several months were found to be firmly anchored by this omental tissue.

From the Department of Physiology, Western Reserve University Medical School, Cleveland, Ohio.

This investigation received financial support from the Council on Pharmacy and Chemistry, American Medical Association.

A preliminary report on this work appeared in the Federation Proceedings, 3: 37, 1944. Received for publication, June 9, 1944.

The coils (Fig. 3) usually employed for the study of pyloric sphincter motility consist of a cross-shaped base of tinned brass 0.25 mm. thick. The three short arms were 5 mm. long, 1 mm. wide and each was pierced at the outer end with a 0.38 mm. hole to be used in suturing the coil in the location desired. The fourth arm was 10 mm. long, 3 mm. wide and was divided into two arms by a slot 1 mm. wide extending into the long arm for 7 mm. The arms were shaped to conform to the surface to which they were to be fastened. The core was a piece of tinned soft iron 1.25 mm. in diameter and 8.5 mm. in length. It was soldered to the base plate in such a manner as to form a 2.5 mm. projecting nib. The coil windings are insulated from the base plate by a thin fiber disk, and from the core by a layer of insulating lacquer.

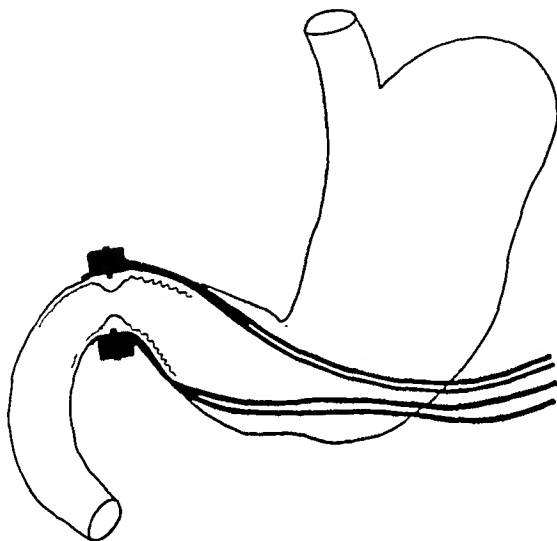


Fig. 1.

The coil proper consisted of approximately 1,000 turns of No. 40 Formex coated copper wire and was 6.25 mm. in diameter and 4.5 mm. thick. The lead wires were made by braiding together 10 strands of No. 40 Formex coated copper wire and three strands of No. 36 stainless steel suture wire. This was encased in $\frac{1}{16} \times \frac{1}{32}$ inch rubber tubing. One end of each rubber tube was slipped over a long arm of the base and secured with silk thread or with lacquer. The lead wires had previously been insulated from the base arm by a bushing made from a short piece of fine French woven catheter. The entire coil and base were coated with several layers of clear Polymerin No. 55* baked at 135° C. for one-half hour, or Bakelite lacquer XV-14633† baked at 135° C. for one hour.

The resistance of each coil was approximately 50 ohms. The inductive resistance offered by a coil to a 60 cycle alternating potential was small and thus the impedance of the coil under operating conditions was also approximately 50 ohms.

When the inductograph was in use, one coil (C_1 , Fig. 2) was made the primary and was energized by connection in series with a suitable variable

*Courtesy of Ault and Wiborg Corporation, Cincinnati, Ohio.

†Courtesy of Bakelite Corporation, Bloomfield, N. J.

resistance (R) to the 1.5 volt tap of a 60 cycle filament transformer (T). The oscillating magnetic field generated by C_1 induced in the secondary coil (C_2) a small alternating potential which in turn was amplified by a radiotronic amplifier (A) having a rated output of 13 watts and a maximum gain of 112 decibels. The amplifier output was arranged to energize the mirror oscillograph (M) of the Viotor type having an impedance of 5000 ohms. A beam of light from the

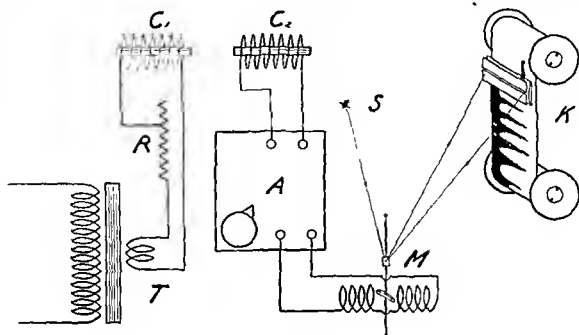


FIG. 2

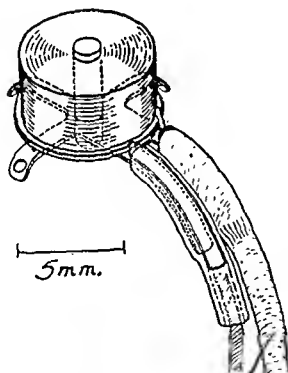


Fig. 3.

slit lamp (S) was reflected from the oscillograph mirror onto the sensitized paper of a photokymograph (K). Since the impressed current was oscillating, the oscillograph produced an oscillating band of light which lengthened as the coils approached each other and shortened as they separated. We customarily recorded only the upper portion of the band and thus obtained a record, the peaks of which indicated approximation of the coils (interpreted as pyloric contraction), while the troughs indicated separation of the coils (pyloric relaxation or opening).

The significance of several possible errors and artifacts was investigated as follows:

1. The passage of peristaltic waves over the pyloric region may rotate the coils about the lateral axis (rocking on the coil base). Table I details the effects obtained in vitro at various interpolar distances by rotating one coil about its base and shows that moderate angular rotation has an essentially negligible influence on the magnitude of the e.m.f. induced in the secondary coil. Furthermore, fluoroscopic observations of animals having implanted coils show that peristaltic contractions and other normal phenomena rotate the coils less than 5° from the longitudinal axis. Thus, the possibility of error due to coil rotation is negligible.

TABLE I
EFFECT OF ROTATING ONE COIL ABOUT LATERAL AXIS

ANGLE	BAND LENGTH WITH INTERPOLAR DISTANCE OF:		
	7.5 MM.	14 MM.	19 MM.
0°	94 mm.	19.5 mm.	16.5 mm.
5°	94	18	16.5
10°	94	18	16.5
15°	91	18	16.5
20°	91	18	16
25°	89	17.5	16
30°	89	17.5	15

2. Increasing the intrapolar distance by lateral displacement of the coils is about as effective as longitudinal displacement in modifying the band length (curve *XF*, Fig. 4). Fluoroscopic examinations of implanted coils at the pyloric sphincter fortunately indicate that in practice, significant lateral displacement does not occur.

3. Placing soft tissue, bone, wood, glass, water, normal saline, barium sulfate, etc., between or around the coils of an inductograph did not modify the magnitude of e.m.f. induced in the secondary coil. Probably this was related to the low ferro-magnetic content of these substances.

4. When the magnitude of current ordinarily employed under experimental conditions was allowed to pass through the primary coil for 30 minutes, no perceptible elevation of coil temperature occurred. Thus, the heat developed in the primary coil probably did not injure the tissue in which it was imbedded or modify its activity.

5. No detectable e.m.f. was induced in the leads of the secondary coil by passing a current of the magnitude ordinarily used in the primary circuit through "dummy" leads placed adjacent to the secondary leads.

6. Cutting of lines of flux by the motion of the coils themselves might induce current. Ordinary sphincter peristalsis, however, would only move the coils approximately 0.5 cm. in 5 to 7 seconds and this interval is too long in comparison to the time required for the primary coil to vary the flux density between zero and its maximum value ($\frac{1}{240}$ second) to induce significant current in the secondary coil. We found that normal peristaltic movements were too slow to induce detectable e.m.f. in the secondary coil by cutting lines of force even when the primary was energized by direct current.

A series of typical calibration curves made in vitro are shown in Fig. 4. They belong to the family of hyperbolic curves having an exponential factor somewhat greater than 2. Each curve has a steep, approximately linear portion and this section of the curve has been used in our experiments.

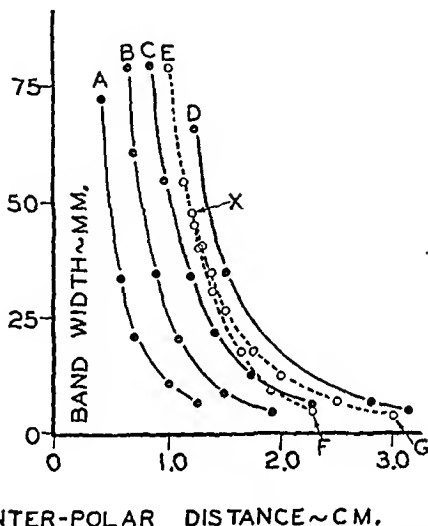


Fig. 4.—In vitro calibration curves of a pair of coils used in a pyloric inductograph showing effects on the band width of the coils when employing a gain control setting of A-5, B-1 or E-15, the effect of increasing the interpolar position at X of 1.2 cm. X F shows the effect of one coil from an original

The characteristic motility of the pyloric sphincter as recorded by the pyloric inductograph consists of rhythmic contractions having a 10.4-14.1 second cycle. In the fed animal, they tend to be more uniform, more continuous, and of greater magnitude than during periods of fasting. A detailed description of this activity and its relation to gastric evacuation will be given elsewhere.

NEW SIMPLE SILVER STAIN FOR DEMONSTRATION OF BACTERIA, SPIROCHETES, AND FUNGI IN SECTIONS FROM PARAFFIN- EMBEDDED TISSUE BLOCKS

GABRIEL STEINER, M.D., AND GRETE STEINER, M.A.
DETROIT, MICH.

IN 1937 a simple method for the demonstration of spirochetes in frozen sections was published.¹ One of the disadvantages of this method was that in steps 8 and 13 a strong heat to the bubbling point had to be used and the presence of the technician was consequently required. A second disadvantage was the use of frozen sections. For these two reasons a silver staining technique for organisms in routine paraffin sections was sought for. The new method given below has been used for the last three years. Sections from the formalin-fixed tissue blocks embedded in paraffin were cut 6 to 7 microns thick and mounted on slides with clean egg albumen. *Zenker-fixed tissue could not be used, nor could celloidin films be used.*

METHOD OF STAINING

1. Deparaffinize sections in the usual manner.
2. Place sections in an aqueous 1 per cent uranium nitrate solution for 3 minutes.
3. Wash twice in distilled water.
4. Immerse in a 1 per cent aqueous solution of silver nitrate (c.p.) for 2 hours in the oven (56 to 58 degrees).
5. Wash thoroughly in distilled water.
6. Dehydrate up to absolute alcohol and place sections in 2.5 per cent alcoholic (absolute) gum mastic solution for 5 minutes.
7. Without washing them transfer sections directly from the gum mastic solution into the reducing solution of hydroquinone (1 Gm. hydroquinone dissolved in 60 c.c. distilled water or equivalents, to which are added 20 c.c. of a 2.5 per cent alcoholic [absolute] gum mastic solution and 20 c.c. of silver nitrate sodium potassium tartrate solution, see below). Mix these three solutions thoroughly by moving the track with the slides up and down for some seconds in the dish. Leave the slides in the reduction mixture for twelve to fifteen minutes until the color of the sections changes to light brown.
8. Wash three times in distilled water, dehydrate, clear in xylol, and mount in Canada balsam.

CHEMICALS

Uranium nitrate, c.p., Baker's analyzed.

Silver nitrate, c.p., Baker's analyzed.

Gum mastic, powdered, Eimer and Amend.

From the Brain Disease Registry, Department of Pathology, Wayne University College of Medicine, Detroit, Michigan.

Received for publication, Mar. 20, 1944.

Hydroquinone, Cenco.

Sodium potassium tartrate powder, c.p.

Xylene, c.p., Baker's analyzed

Distilled water or, preferably, twice distilled water for steps 3 and 5 and for preparing the silver nitrate solutions.

SOLUTIONS

A. Gum Mastic Solution—The solution is made by adding 2.5 Gm. of powdered gum mastic to 100 c.c. of absolute alcohol. It is dissolved by stirring with a glass rod and is left standing overnight. The solution is filtered several times until clear.

B. Reduction Mixture.—The silver nitrate sodium potassium tartrate solution is prepared as follows: 2 Gm. of silver nitrate (c.p.) are dissolved in 1,000 c.c. of boiling distilled water. When the chemical is dissolved and the water is boiling, 1.65 Gm. of powdered sodium potassium tartrate is quickly added until the white precipitate changes to gray. Then the solution is filtered while hot into an amber bottle with glass stopper. The silver nitrate sodium potassium tartrate solution is poured into the alcoholic gum mastic solution and not vice-versa; equal parts, 20 c.c. each, of silver nitrate sodium potassium tartrate solution and of 2.5 per cent alcoholic gum mastic solution are used. A milky fluid results which is the 40 c.c. added to the dish containing the 60 c.c. of hydroquinone solution (1 Gm. of hydroquinone dissolved in 60 c.c. of twice distilled water) and the slides

The silver nitrate sodium potassium tartrate solution is the same as that used in the silver mirror method for smears.²

The final concentrations in the reduction mixture are

hydroquinone 1 per cent,
gum mastic 0.5 per cent, and
silver nitrate less than 0.04 per cent,

because the precipitate formed in the sodium potassium tartrate solution has been eliminated by filtration.

The 2.5 per cent alcoholic gum mastic solution, the 1 per cent aqueous uranium nitrate solution, the 1 per cent aqueous silver nitrate solution, and the silver nitrate sodium potassium tartrate solution can be kept indefinitely in stock, the last two in amber bottles with glass stoppers in the refrigerator. The hydroquinone solution has to be prepared each time shortly before use.

Instead of staining dishes with removable racks (20 slides may be accommodated if the slides are placed back to back in pairs), Coplin jars can be used which accommodate 8 slides, three pairs in the inner grooves and one slide in each outer groove.

In using Coplin jars care has to be taken that the concentrations of the solutions are equivalent to those previously given; for example, hydroquinone solution has to be made up by dissolving 0.5 Gm. in 30 c.c. of distilled water; consequently 10 c.c. of 2.5 per cent alcoholic gum mastic solution and 10 c.c. of silver nitrate sodium potassium tartrate solution are used. In our laboratory, staining dishes of 200 c.c. capacity were used so that 2 Gm. of hydroquinone were dissolved in 120 c.c. of distilled water; 40 c.c. of 2.5 per cent alcoholic gum

A HOMEMADE MECHANICAL PIPETTE

M-40753 S/Sgt. M. S. SMITH, No. 1 CANADIAN BASE LABORATORY,
R.C.A.M.C., C.A. (O)

AN AUTOMATIC pipette is almost indispensable in a laboratory handling large numbers of Kahn and Wassermann tests. When the serological service was instituted in the Canadian Army Overseas, it was found impossible to produce an automatic or a mechanical pipette, and the problem arose of making one from the material at hand. The following is a description of an automatic pipette designed and built by the writer, and in use for the past year. This machine is easy to handle and delivers accurately amounts from 0.25 c.e. up.

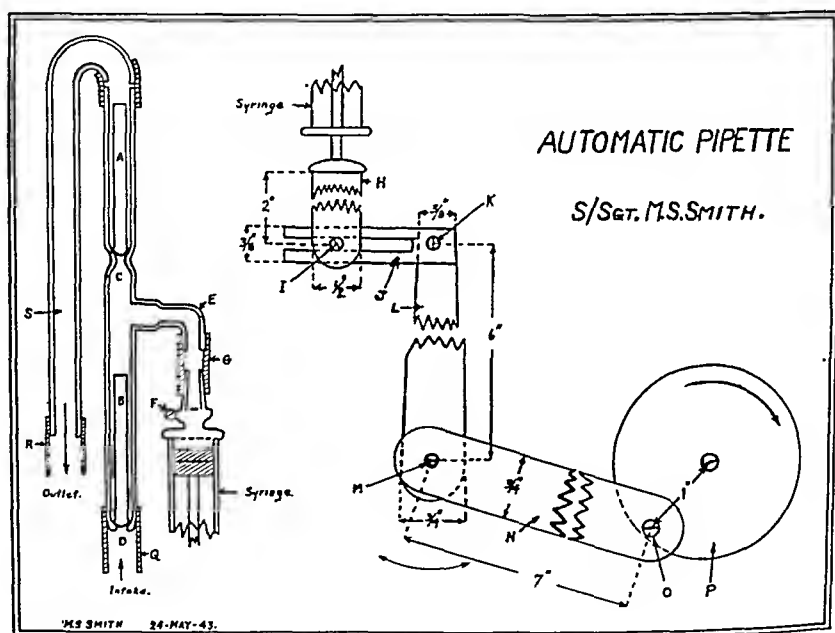


Fig. 1.

The motor is the type used for the Van Slyke apparatus, is geared, and has a variable speed. In the case of this machine, it was necessary to use electric light bulbs in series to cut down the voltage from 220 v to 110 v. The flywheel *P* has an eccentric and is attached by a series of lever arms to the plunger of a 2 c.c. Record syringe. The syringe may be adjusted for amounts from 0.25 c.e. up by moving the bearing *I* along the slot in *J*. The flow through the pipette is directed by valves *A* and *B*. On the downstroke of the plunger, *A* closes and *B* opens; on the upstroke the reverse occurs, ensuring a regular even flow of fluid along *S* to rubber pressure tubing and thence to test tubes. Details of the

Received for publication, March 25, 1944.

machine may be better understood by reference to Fig. 1. The flywheel *P* moves the syringe plunger through the medium of the members *T*, *L*, and *N* which are made of strap iron and have bearings at *O*, *M*, *K*, and *I*. The syringe

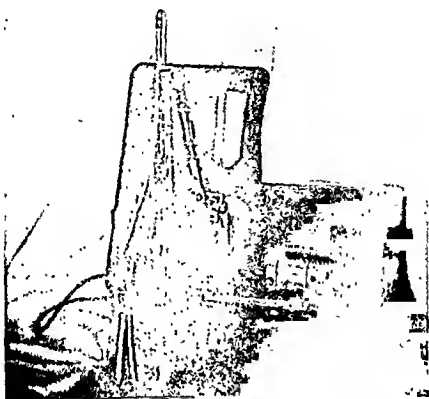


Fig. 2



Fig. 3.

nozzle is hinged at *F* by a piece of brass rod $\frac{1}{8} \times 1\frac{1}{2}$ inches soldered to it. At *G* is a piece of rubber pressure tubing connecting the syringe to the *T* tube and valve system. In the valve system a good grade glass *T* tube $\frac{3}{16}$ inch inside

diameter is used. One end is partially closed in a flame for valve seat *D*. Valve *B* is made first, before the tube is drawn out for valve seat *C*. To make the valves, take a piece of glass rod about 12 inches long that will have about $\frac{1}{16}$ inch clearance when inserted into the *T* tube. Smooth one end in a flame and insert into the *T* tube for a trial fit. The valve and valve seat are ground to fit with pumice and turpentine, using a rotating motion. When a fit is obtained, the rod is then cut about $1\frac{1}{2}$ inches from the ground end and this shorter piece constitutes the valve *B*. After valve *B* is in place, the other end of the *T* tube is carefully drawn out in a flame for the valve seat *C*, and valve *A* is ground to fit in the same way as valve *B*. The stem *E* of the *T* tube is then bent downwards to connect with the syringe system by means of rubber pressure tubing at *G*.

The motor is covered with a plain box for protection and to give support for bearing *K* and to the valve assembly (Fig. 2). The whole apparatus is constructed from material at hand and is a very useful efficient machine (Fig. 3).

CHEMICAL

DETERMINATION OF TOTAL AND FREE CHOLESTEROL

A. G. SHEFTEL, M.D., BEVERLY HILLS, CALIF.

NUMEROUS methods are available for the determination of total cholesterol, most of them based on the Liebermann-Burchard reaction, in which a blue color develops when cholesterol is treated with acetic anhydride and concentrated sulfuric acid. The majority of these tests are concerned with various steps of extraction of the cholesterol from the blood.

The color produced in the Liebermann-Burchard reaction is evasive and of short duration. The complete intensity is attained in a few minutes, depending on temperature and light factors. The peak of the color intensity lasts but a few minutes and then fades rather rapidly. It is essential, therefore, to take colorimetric readings of the unknown and the standard at the same time. When several determinations have to be made, the later colorimetric readings may be outside the peak of the curve, giving an error in results, especially when a permanent standard is used, or a permanent curve is plotted with a photoelectric colorimeter.

The method herein described produces a more stable color. By adding glacial acetic acid to the Liebermann-Burchard reagents, the color develops much more slowly, and the peak of color intensity is of much longer duration, very gradually fading away. Chart 1 is a graphic presentation of the color development with and without the addition of glacial acetic acid, as recorded by a photoelectric colorimeter (Klett), under identical conditions of temperature and light. The heavy line is a record of results with the use of glacial acetic acid; the light line, without. The abscissa represents the time and the ordinate, the colorimetric readings. It can be seen from the chart that without glacial acetic acid the color develops rapidly, reaching its maximum intensity in nine minutes. The peak of the color intensity remains for five minutes, then rapidly fades. Twenty minutes after reaching its maximum intensity, the color has faded 18 per cent. With the addition of glacial acetic acid, however, the color reaches its maximum intensity in twenty-one minutes, remains at a peak for fourteen minutes, then begins to fade much more slowly. Twenty minutes after reaching its maximum intensity, the color has faded only 1 per cent. The color produced with glacial acetic acid, although slightly less intense at its peak than the color produced without glacial acetic acid, nevertheless is sufficiently deep to make accurate determinations even with small amounts of cholesterol present.

Several experiments were made with various stocks of glacial acetic acid as follows: (1) a new bottle; (2) a bottle that had been kept open for several weeks; (3) glacial acetic acid to which 1 per cent water was added. Invariably, all colorimetric readings were the same.

EXTRACTION OF THE CHOLESTEROL

Various methods were studied for the extraction of cholesterol from the blood, and it was found that the most thorough and expedient method which did not require special apparatus was to extract the lipids from the blood by means of acetone-alcohol mixture, evaporate the extract, and then isolate the cholesterol from the residue with chloroform.

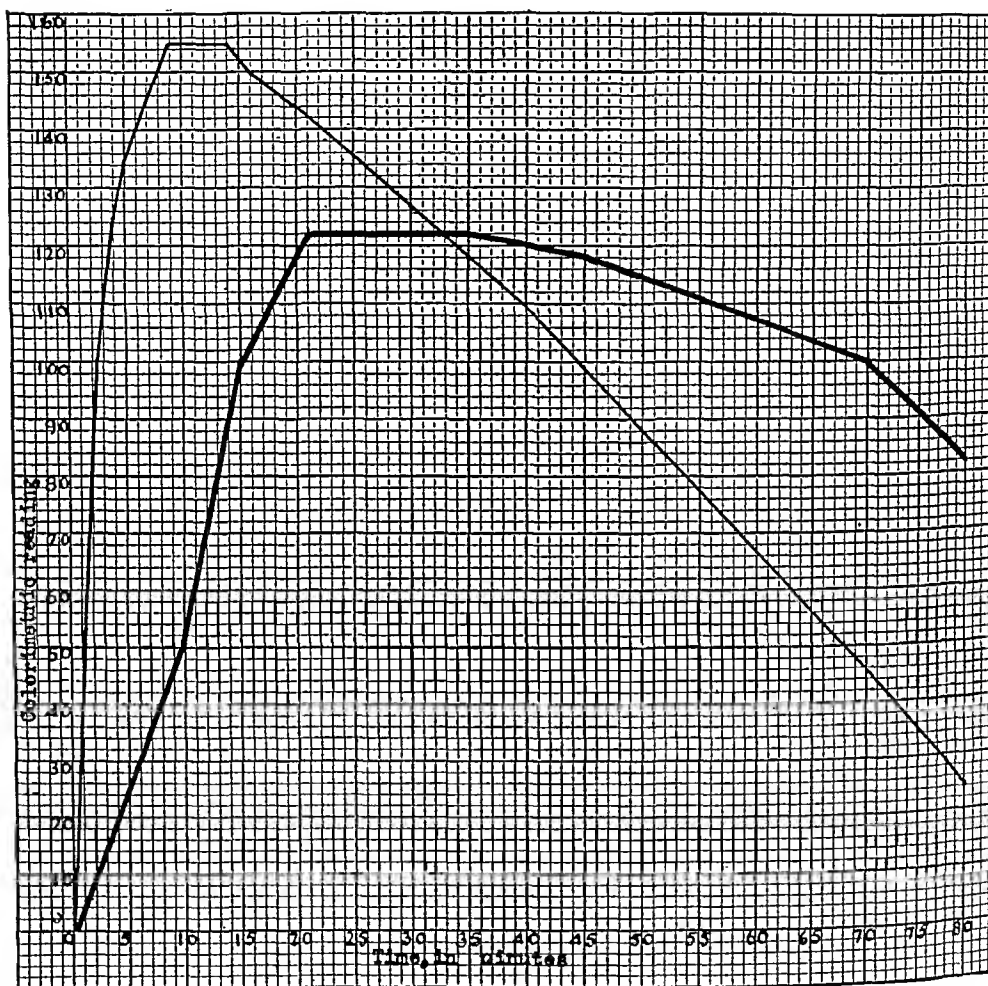


Chart 1.—Color development with and without glacial acetic acid. Heavy line with glacial acetic acid, light line without.

PROCEDURE FOR TOTAL CHOLESTEROL

Reagents.—

1. Acetone-alcohol, equal parts of acetone and absolute alcohol.
2. Chloroform, C.P., reagent.
3. Glacial acetic acid.
4. Acetic anhydride.
5. Concentrate sulfuric acid.

6. a. Stock cholesterol solution, containing 1 mg. of cholesterol in 1 c.c. of chloroform.
- b. Working standard: dilute 1 c.c. of the stock to 10 c.c. volume with chloroform.

Technique.—

Introduce into a Pyrex test tube graduated at 5 c.c., or into a graduated centrifuge tube, 4 c.c. of acetone-alcohol mixture. Add to it slowly, 0.5 c.c. of oxalated blood. When determination of free cholesterol is also required, use a test tube graduated at 10 c.c.; add to it 8 c.c. of acetone-alcohol and 1 c.c. of oxalated blood. Immerse test tube in hot water of a temperature near boiling point until the solution in the test tube begins to boil. Then remove the test tube from the water bath and stir contents thoroughly with a stirring rod. Repeat this boiling process twice more. Let stand two or three minutes, and then add acetone-alcohol mixture to the 5 c.c. mark (10 c.c. mark for free cholesterol). Stir thoroughly. Insert a cork into test tube, and centrifuge for about three minutes at moderate speed. Decant supernatant fluid into a clean test tube. Use 3 c.c. of this extract for the total cholesterol; 4 c.c. for free cholesterol. Where total cholesterol alone is required, 3 c.c. can easily be pipetted off without transferring into another test tube. Transfer 3 c.c. of the extract into a 100 c.c. dry Pyrex beaker and evaporate slowly, just to dryness. To prevent too rapid evaporation, it is advisable to place a piece of asbestos on top of the electric plate. After the residue in beaker is dry, add to it about 5 c.c. of chloroform and evaporate slowly to about half volume. Transfer the extract to a dry test tube graduated at 5 c.c. Repeat extraction process until the 5 c.c. mark is reached. Let stand for a few minutes to cool; then add 1 c.c. of glacial acetic acid, 2 c.c. of acetic anhydride, and 0.2 c.c. of concentrated sulfuric acid. Insert rubber stopper, invert twice, and place tube immediately into a water bath of 23° C. for twenty-five minutes. Examine in colorimeter. A red filter facilitates matching of colors. In order not to have excessive variations in light, it is advisable to develop the color in the same corner of the laboratory. We have found that a brown glass jar is very useful for such a water bath. Prepare the standard by adding 5 c.c. of the working standard, 1 c.c. of glacial acetic acid, 2 c.c. of acetic anhydride, 0.2 c.c. of concentrated sulfuric acid; treat this mixture in the same way as the unknown. This standard represents 167 mg. cholesterol per 100 c.c.

TABLE I

NUMBER OF TEST	PRESENT METHOD	BLOOR METHOD
	TOTAL CHOLESTEROL IN MG. PER 100 C.C.	TOTAL CHOLESTEROL IN MG. PER 100 C.C.
1	162	158
2	213	214
3	186	192
4	204	202
5	354	364
6	180	184
7	421	417
8	167	167
9	210	212
10	208	206
11	196	198
12	210	206

Table I shows comparative results obtained with the method herein described and the Bloor method. In the latter only two tests were run at the same time, and a fresh standard was made for each determination. The color of the unknown and the standards were developed under identical conditions of temperature and light. The extraction procedure in this method does not produce any extraneous color, since there is a tendency in this method to give slightly lower values, as compared with other methods employing the alcohol-ether extraction.

METHOD FOR FREE CHOLESTEROL

Reagents.—

Above reagents as employed in method for Total Cholesterol, plus:

1. Digitonin solution: 1 per cent digitonin in 95 per cent alcohol.
2. Ether-acetone mixture, 2 parts to 1.
3. Standard solution containing 0.5 mg. cholesterol in 1 c.c. glacial acetic acid.

Technique.—

To 4 c.c. of acetone-alcohol extract, add 1 c.c. of digitonin solution and 1 c.c. of distilled water. Mix, and allow to stand for about forty minutes. Centrifuge for ten minutes at high speed. Decant supernatant fluid and let the inverted test tube stand over a piece of filter paper for two to three minutes. Wipe mouth of test tube with filter paper, and add 10 c.c. of ether-acetone mixture, washing sides of the test tube well with this mixture. Stir precipitate and centrifuge for ten minutes at high speed. Decant supernatant fluid, drain again as before, and add again 10 c.c. of the ether-acetone mixture. Stir precipitate and centrifuge for ten minutes at high speed. Decant supernatant fluid, dry mouth of test tube with filter paper and immerse test tube in warm water at 50° C. for a few minutes (to dry precipitate). Now add 3 c.c. of glacial acetic acid, immerse in water heated to 70° or 80° C. for a few minutes, or until the precipitate is completely dissolved. Cool the test tube. Now add 2 c.c. of acetic anhydride, 0.2 c.c. of concentrated sulfuric acid, stir thoroughly, and allow to stand in a water bath at 23° C. for 1½ hours. Examine in colorimeter.

PREPARATION OF STANDARD

To 1 c.c. of standard solution containing 0.5 mg. of cholesterol, add 2 c.c. of glacial acetic acid, 2 c.c. of acetic anhydride, and 0.2 c.c. of concentrated sulfuric acid. Stir thoroughly and allow to stand for 1½ hours in a water bath of 23° C. This standard represents 125 mg. of free cholesterol. To determine cholesterol esters, deduct free cholesterol from the total cholesterol.

SUMMARY

Blood is extracted with acetone-alcohol 1:1, the extract evaporated and extracted with chloroform. Glacial acetic acid is added to the chloroform extract. The addition of the glacial acetic acid produces a more stable color of Liebermann-Burchard reaction. The acetone-alcohol extract is also being used for the determination of free cholesterol.

SULFONAMIDE DETERMINATION IN BLOOD USING XYLENESULFONIC ACID AS A PROTEIN PRECIPITANT

S/SGT. R. E. FLORIN, MED. DEPT. A.U.S. ARMY
FIRST LIEUT. R. M. SILVERSTEIN, S.C., A.U.S.

SULFONAMIDES are ordinarily determined in blood according to the method of Bratton and Marshall using trichloroacetic acid as the protein precipitant. Because of the acute shortage of trichloroacetic acid in the Southwest Pacific Area, a method has been devised in which the protein is precipitated by xylenesulfonic acid. The possibilities of this reagent were suggested by the use of p-toluenesulfonic acid in an older method of Marshall.¹ The synthesis of p-toluenesulfonic acid was precluded by the scarcity of toluene. Xylene, on the other hand, is readily available, as is technical sulfuric acid.

METHOD

Protein is precipitated from blood with a mixture of xylenesulfonic and sulfuric acids, and sulfonamides are determined by coupling in alcohol solution with N-(1-naphthyl)—ethylenediamine dihydrochloride according to a modified Bratton-Marshall method.²

REAGENTS

- a. Xylenesulfonic acid. Place 30 c.c. xylene and 40 c.c. concentrated sulfuric acid (technical grade is satisfactory) in a 125 c.c. flask. Warm to start the reaction and shake at intervals until a nearly clear solution is obtained (about 30 minutes). Separate the small amount of unreacted xylene. Dilute to 250 c.c. with water. Bring to a boil in a 1,000 c.c. beaker. Cool, and add slowly, with stirring, a suspension of 34 Gm. calcium hydroxide in 50 c.c. water. Boil for one minute, and filter with suction. Wash thoroughly with three 10 c.c. portions of hot water, stirring up the mass each time. Dilute the filtrate to 280 c.c. Allow to stand for two days and decant from the precipitant formed. This gives approximately a 20 per cent solution.
- b. Sulfuric acid. N/12.
- c. Xylenesulfonic-sulfuric acid mixture. To 15 volumes N/12 sulfuric acid (b) add 4 volumes xylenesulfonic acid (a).
- d. Sodium nitrite 0.1 per cent. Freshly prepared.
- e. Ethyl alcohol. 95 per cent.
- f. N-(1-naphthyl)—ethylenediamine dihydrochloride, 0.1 per cent. Keep in refrigerator in brown bottle.
- g. Stock standard sulfonamides. 100 mg. per liter for all except sulfadiazine (50 mg. per liter). Keep in refrigerator.
- h. Strong working standard. 1 c.c. stock (2 c.c. for sulfadiazine) diluted to 20 c.c. with xylenesulfonic-sulfuric acid mixture (c).

- i. Weak working standard. 1 c.c. stock (2 c.c. for sulfadiazine) diluted to 50 c.c. with xylenesulfonic-sulfuric acid mixture (c).

PROCEDURE

Add 1 c.c. oxalated blood dropwise with shaking, to 15 c.c. N/12 sulfuric acid (b). Allow to lake for two minutes. Then add, dropwise with shaking, 4 c.c. xylenesulfonic acid (a). Filter. One cubic centimeter oxalated blood may be added alternatively to 19 c.c. xylenesulfonic-sulfuric acid mixture (c). To 10 c.c. filtrate and to 10 c.c. each of the strong and weak standards, add 0.5 c.c. sodium nitrite solution (d). Mix and allow to stand three minutes. Add 5.0 c.c. ethyl alcohol (e) and mix. Add 0.5 c.c. N-(1-naphthyl)-ethylenediamine dihydrochloride (f) with vigorous swirling. Allow to stand at least a minute and compare in the colorimeter, using the closer matching standard. The colors are stable for several hours.

CALCULATIONS

Weak standard:

$$S/U \times 4 = \text{mg. per 100 c.c. blood.}$$

Strong standard:

$$S/U \times 10 = \text{mg. per 100 c.c. blood.}$$

Recovery experiments and comparative studies were made employing both trichloroacetic acid and xylenesulfonic acid as precipitating agents (Table I).

TABLE I

BLOOD LEVELS (IN MG. PER 100 C.C.) OF FREE SULFONAMIDES OBTAINED IN RECOVERY EXPERIMENTS AND COMPARATIVE STUDIES USING TRICHLOROACETIC ACID AND XYLENESULFONIC ACID AS PRECIPITATING AGENTS

SULFONAMIDE	PREPARED CONCENTRATION	TRICHLOROACETIC ACID METHOD	XYLENESULFONIC ACID METHOD
Sulfadiazine	10.0	9.5	9.6
	5.0	4.2	4.0
	----	5.9	5.7
	----	10.1	9.5
	----	4.3	4.2
Sulfathiazole	10.0	9.4	9.2
	10.0	--	9.1
	10.0	--	8.9
	10.0	--	9.4
	10.0	--	9.0
	10.0	--	9.4
	5.0	--	3.9
Sulfaguanidine	10.0	9.4	9.5
	5.0	4.1	4.2
	2.0	--	2.0

SUMMARY

An alternate method for determining blood sulfonamides has been described. The method offers a substitute protein precipitant, prepared from readily available laboratory reagents. Results are comparable with those obtained by the standard trichloroacetic acid method.

REFERENCES

1. Marshall, E. K., Jr.: Determination of Sulfonamide in Blood and Urine, *J. Biol. Chem.* 122: 263, 1937.
2. Lee, S. W., Hannay, N. B., and Hand, W. C.: Rapid and Accurate Micro Method for Estimation of Sulfonamides, *Science* 97: 359, 1943.

another is the rubber joint *B* by which the arms of the siphon are adjusted in length until the fluid columns balance when *T* is removed. Before use the siphon is filled with wash fluid by suction at *D*, preferably by mouth, by way of a tube with mouthpiece.

With tube *T* in position the siphon flows; the flow may be accelerated by suction, stopped suddenly, or even reversed by pressure at *D*. Because of the balanced siphon, suction by mouth requires little effort and gives more control than is possible through any mechanical source. Just before the tip disengages the fluid, the suction is removed.

The decanter is best mounted by clamping the neck of flask *E* to a ring stand. The centrifuge tube *T* is held in place and manipulated mechanically by an adjustable screw or simply steadied by thumb and forefinger against a ring stand.

SUMMARY

A micro-decanter for the separation of the wash fluid from a metallic precipitate in centrifuge washing is described. Use of a balanced siphon rather than power suction gives delicate control so that accidental removal of floating flakes of the precipitate is avoided.

BOOK REVIEWS AND NOTICES

The Riddle of Cancer. By Charles Oberling, M.D., translated by William H. Woglom, M.D. Yale University Press, New Haven, Conn. Price \$3.00. Cloth with 196 pages.

Aside from two introductory chapters this book of 196 pages is divided into three parts: first, an unbiased exposition of three theories of the nature of cancer; second, a discussion of modern experimental research in cancer; and third, a vigorous defense of the author's theory of the cause of cancer—that it is caused by viruses.

As would be expected and is natural, there is considerable tendency to emphasize the contributions of the French, to the exclusion (perhaps only nonmention) of others. The position of Virchow, Cohnheim, and others, however, is clearly made and the only charge is pardonable provincialism.

Whether or not the laity can derive instruction and information must remain for the laity to determine for themselves. A fair statement is on the paper jacket. "It is a book for laymen as well as doctors since it is written with a simplicity that does not flinch from using technical terms or defining them." There can be little question of the simplicity and logic of the discussions (which is as much to the credit of the translator as of the author). On the other hand every page is sprinkled with scientific terms, some of which are not defined. A guess might be that the educated lay person will find the style and context sufficiently intriguing to stimulate him to read. In sarcasm, the author is at his best. For example on page 34 of the section devoted to the microbial or parasitic hypothesis of cancer: "Such a spectacle was presented hardly eight years ago, when it was announced to an astonished world that tomatoes are the cause of cancer. Not only announced but proved! For the injection of tomato juice into the peritoneal cavities of rats was said to have been promptly followed by sarcomas, and photomicrographs were offered in support of the assertion. Well, it was difficult to incriminate such a common article of diet, so a microorganism was sought, and as though by magic one appeared: an inoffensive bacillus of the subtilis group that everyone carries in his skin. But there, it was said, were the proofs; this microbe, isolated from tomato juice, had been obtained in pure culture, and produced sarcoma when inoculated into rats. No one, of course, was able to confirm these experiments and both tomato and subtilis were thereupon restored to favor, but neither has anyone ever discovered how these 'tomato sarcomas' were produced." A close second in charm of style is the occasional side remark revealing the philosophy of the author. At the end of a discussion of experiments and theories on page 16: "Ideas have arisen, temuous at first, later taking form, to crystallize at last in a number of definite conceptions. Not yet having been consecrated by experiment, these remain only working hypotheses, but even so they have been immensely valuable. If some inaccuracies, and even errors, must be admitted, all hold a

another is the rubber joint *B* by which the arms of the siphon are adjusted in length until the fluid columns balance when *T* is removed. Before use the siphon is filled with wash fluid by suction at *D*, preferably by mouth, by way of a tube with mouthpiece.

With tube *T* in position the siphon flows; the flow may be accelerated by suction, stopped suddenly, or even reversed by pressure at *D*. Because of the balanced siphon, suction by mouth requires little effort and gives more control than is possible through any mechanical source. Just before the tip disengages the fluid, the suction is removed.

The decanter is best mounted by clamping the neck of flask *E* to a ring stand. The centrifuge tube *T* is held in place and manipulated mechanically by an adjustable screw or simply steadied by thumb and forefinger against a ring stand.

SUMMARY

A micro-decanter for the separation of the wash fluid from a metallic precipitate in centrifuge washing is described. Use of a balanced siphon rather than power suction gives delicate control so that accidental removal of floating flakes of the precipitate is avoided.

to psychiatry and should not attempt to make the student depend on it as a therapeutic guide. On page 32 the author states that in the case of paresis, "The serology alone is so diagnostically decisive, that in an untreated case, the diagnosis may be made in the laboratory without seeing the patient." This statement encourages a student to rely too exclusively on laboratory techniques.

In spite of these limitations this introduction to psychiatry is one of the most useful outlines, available

EDWIN F. GILDEA, M.D.

Homicide Investigation. LeMoyne Snyder, M.D., Medicolegal Director, Michigan State Police. Charles C. Thomas, Springfield, Ill., 1944. Price \$5.00. Cloth with 287 pages.

Written primarily for coroners and police officers, this book has much to recommend it as a practical reference and guide to physicians who occasionally participate in the investigation of suspected homicide. Excellently printed and well illustrated, the book covers both general and specific considerations of the medical and nonmedical phases of investigation.

Special chapters on the interrogation of the criminal, investigation of highway accidents, and the collection and preservation of firearms evidence were contributed by other members of the Michigan State Police and the Chicago Scientific Crime Detection Laboratory. The remaining chapters deal with examination of the scene, examination of blood stains, the detection of alcohol, examination for suspected assault, deaths from asphyxia, drowning, burning, poisoning, blunt and penetrating violence, criminal abortion, and gunshot wounds.

Appearing about the time when the "Report of the Committee of the American Medical Association to Study the Relationship of Medicine and Law" was published in the *Journal of the American Medical Association* (125: 577-583, June 24, 1944), it becomes obvious that there is a definite need of more general interest on the part of the medical profession for coordinated medical and legal activity. The author, who is also a member of the American Bar Association, is competent to present his views to both public officials and physicians. Exasperated at being confronted frequently in his work as a consultant by inadequate and misdirected investigation on the part of those charged with collecting the necessary preliminary evidence, Dr. Snyder has attempted to provide coroners, police officers, and others with a useful, simple handbook to serve as a pattern for proper investigation.

He has succeeded well in his purpose. The book is written for the most part in nontechnical terms. It shares the fault of any book that aspires to simplification; experts in the field will find numerous general conclusions that need qualification, but this does not detract from the value of the book. Laboratory procedures are referred to in general terms indicating the type of information which may be expected so that the coroner or police officer will not be tempted to carry out procedures beyond his scope.

The book includes over a hundred photographic illustrations, many of which are excellent. The quality of the reproductions of the color photographs is perhaps out of proportion to the added expense.

HERBERT S. BREYFOGLE.

The Diabetic Life, Its Control by Diet and Insulin. By R. D. Lawrence, M.A., M.D., F.R.C.P. (London), Physician in Charge, Diabetic Department, King's College Hospital, Late Chemical Pathologist and Lecturer, King's College Hospital. 13th Edition, Cloth with 228 pages and 18 illustrations. Price \$4.00. The Blakiston Company, Philadelphia, Pa.

Lawrence is a well-known specialist in the treatment of diabetes, and this little book summarizes what he has found to be important and practical in the treatment of the disease. The information given is sound, and the directions are explicit. It is what it purports to be, "a concise practical manual for practitioners and patients." Since the first edition, published in 1925, the methods of this London physician have been widely followed in Great Britain, and have also been used by many people in this country. A "War-Time Supplement" heads the new edition, with special instructions and diets for war-torn England. For example, the proper use of the extra protein and fat rations granted to diabetics is outlined. Because of the scarcity of oranges, apples, tomatoes, and lettuce (all diabetic stand-bys), cabbage, turnips, carrots, celery, leeks, and watercress are recommended, and other similar practical substitutions are described. Lawrence's "Line-Ration" scheme of food measurement has always seemed to add to the complication of diet calculation. He seems to be abandoning it in favor of a "Five-Gram" diet scheme. American patients and physicians will no doubt continue to use the simpler carbohydrate-content food-group plan, and the food tables given in this book will not, therefore, be useful to the majority of patients and physicians in the United States.

Physicians interested in the treatment of diabetes will profit from the practical discussion of details of management given, but because of the difference in the American scheme of carbohydrate computation in the diet, other manuals available will prove of greater usefulness to diabetic patients in this country.

CYRIL M. MACBRYDE.

Elimination Diets and the Patient's Allergies: A Handbook of Allergy. By Albert H. Rowe, M.D., Lecturer in Medicine, University of California Medical School, San Francisco, California; Consultant in Allergic Diseases, Alameda County Hospital, Oakland, California. Lea and Febiger, Philadelphia, 1944, Second Edition, thoroughly revised. Price \$3.50. Cloth with 256 pages.

The author of the book is a notable pioneer in the study of food allergy. He has made great contributions to the field of allergy, especially in the use of trial diets for diagnostic and therapeutic aids.

The book covers many symptoms and manifestations of allergy and attempts to correlate them with dietetic control. The main theme of the book is the dietary management of allergy. Rowe emphasizes, as he has done in previous books, the fallibility of skin tests, especially when chronic or delayed type of allergy is present. He very aptly stresses the importance of total exclusion of omitted foods while food allergy is being studied since it must be assumed that maximum food allergy is present. This practical point is overlooked by many who feel that a small amount of a forbidden food will do no

harm. The need of using a trial diet for more than a few days is emphasized, particularly in patients who have allergy of long standing.

Dr. Rowe expresses the fact, well known among allergists, that a period of observation must be long enough to determine whether freedom from symptoms is due to the use of a given diet or to an ordinary remission. He feels that a diet, to be of proved value, should relieve the patient longer than the longest previous symptom-free period. He stresses the necessity of detailed menus to assure proper nutrition during trial diet periods. He gives many standardized menus and mentions the difficulty of securing some of the suggested foods during wartime. Although the attempt is made to standardize trial diet usage, he does feel that a given diet should be modified by definitely positive skin reactions as well as the patient's history of disturbance from a given food.

The book is easily read. Although it discusses various etiologic agents and the clinical manifestations of allergy, its chief value lies in the emphasis it places on food in the etiology and therapeutics of allergy. The book should be studied carefully by all who practice allergy.

C. MALONE STROUD.

Principles and Practices of Inhalational Therapy. By Alvan L. Barach, M.D., Associate Professor of Clinical Medicine, Columbia College of Physicians and Surgeons; Assistant Attending Physician, Presbyterian Hospital. J. B. Lippincott Company, Philadelphia, Pa. Price \$4.00. Cloth with 315 pages and 59 illustrations.

Dr. Barach qualified himself as the author of this handbook by devoting years of study analyzing the techniques and rationale of inhalation therapy. He has done an admirable job of summarizing his own observations and those of other investigators in a thoroughly readable form. After presenting the historical and physiologic background of inhalation therapy, he systematically discusses its use in the treatment of thirty different conditions. A few of these are acute altitude sickness, pneumonia, pulmonary edema, congestive heart failure, shock, coronary thrombosis, emphysema, bronchial asthma, war gas poisoning, and blast injuries of the lungs. Each section begins with a statement of the pathologic physiology of the disease state and an analysis of the rationale for using inhalation therapy. There is a valuable chapter on oxygen poisoning which calls attention to the apparent ability of human subjects to withstand higher concentrations of oxygen than those tolerated by most experimental animals. Barach suggests that the greater tolerance may be due to the fact that patients who breathe high concentrations of oxygen have intermissions during which the mask is changed or their faces washed. The detailed discussion of methods of inhalation therapy contains much practical information as, for instance, the necessary minute flow of oxygen to attain different concentrations of the gas in inspired air, when face masks are used.

The book is beautifully printed, well illustrated, adequately indexed, and contains a good bibliography. As a minor criticism, it should be pointed out that many fundamental investigations done on animals are reviewed only very briefly. The author avoids, however, the mistake of being too enthusiastic in his claims for the possible benefits of inhalation therapy.

C. V. M.

The Analytical Chemistry of Industrial Poisons, Hazards and Solvents. By Morris B. Jacobs, Ph.D., Senior Chemist, Department of Health, City of New York, 1928-; Director of Gas Reconnaissance, Gas Defense Service, New York City Citizens Defense Corps; formerly Lt. U. S. Chemical Warfare Service Reserve. Second revised reprint. 1944, Interscience Publishers, Inc., New York, N. Y. Price \$7.00. Cloth with 661 pages.

The Diet Therapy of Disease. By Dr. Louis Pelner, Assistant Attending Physician, Long Island College Hospital, Greenpoint Hospital, and Brooklyn Cancer Institute; Gastroscopist; Beth Moses Hospital; Lecturer, Postgraduate Course in Gastroenterology, under the charge of Dr. S. A. Seley. Personal Diet Service, New York, N. Y., 1944. Price \$3.75. Cloth with 143 pages.

A Manual of Physical Therapy. By Richard Kovacs, M.D., Professor of Physical Therapy, New York Polyclinic Medical School and Hospital; Attending Physical Therapist, Manhattan State, Harlem Valley State, Columbus, and West Side Hospitals; Visiting Physical Therapist, New York City Department of Correction Hospitals; Consulting Physical Therapist, New York Infirmary for Women and Children; Mary Immaculate Hospital, Jamaica, New York, St. Charles Hospital, Jefferson, L. I., Hackensack Hospital, Hackensack, N. J. Third edition, thoroughly revised, formerly published under the title "Physical Therapy for Nurses" Lea & Febiger, Philadelphia, Pa., 1944. Price \$3.25. Cloth with 309 pages and 118 engravings.

The Principles and Practice of Medicine. By Henry A. Christian, A.M., M.D., LL.D. (Hon.) Sc.D., Hon. F.R.C.P. (Can.), F.A.C.P., Hersey Professor of the Theory and Practice of Physic, Emeritus, Harvard University; Clinical Professor of Medicine, Tufts College Medical School; Physician in Chief, Emeritus, Peter Bent Brigham Hospital; Visiting Physician, Beth Israel Hospital, Boston. Fifteenth edition, D. Appleton-Century Co., New York, N. Y. Price \$9.50. Cloth with 1,498 pages.

The Treatment of Peptic Ulcer. By George J. Heuer, M.D., Professor of Surgery of Cornell University Medical College and Surgeon-in-Chief of the New York Hospital. J. B. Lippincott Company, Philadelphia, Pa. Price \$3.00. Cloth with 118 pages.

The Journal of Laboratory and Clinical Medicine

Vol. 29

SEPTEMBER, 1944

No. 9

CLINICAL AND EXPERIMENTAL

SULFONAMIDE-RESISTANT GONORRHEA TREATED WITH UREA AND SULFONAMIDE BY MOUTH

MAJOR MAURICE A. SCHNITKER, M.C., A U.S., AND
CAPTAIN CHARLES D. LENHOFF, M.C., A.U.S.

AS EFFICACIOUS as the sulfonamides have been in the treatment of gonorrhea, the problem of sulfonamide resistant cases in the Armed Forces, until recently, has been an important one. It constituted as many as 10 to 20 per cent of cases. Penicillin, however, has now made gonorrhea in any of its forms curable in practically all cases.

But before penicillin became available to us, sulfonamide-resistant cases were frequently difficult to treat and resulted in much loss of time from duty. In an attempt to find a more successful method of treatment for these cases than anterior urethral instillations of silver salts and a safer method than fever therapy we were encouraged from the following considerations to try urea by mouth.

After D. D. Woods of London¹ and others had demonstrated that para-aminobenzoic acid, peptone, tissue extracts, etc., may be inhibitors of sulfonamide activity, further studies showed that such substances as azochloramide,² certain purines,³ and urea may behave as *anti-inhibitors*. Such a substance as urea may act in some way to prevent the inhibitors from interfering with the sulfonamide action.

With this newer knowledge, several investigators have tried urea combined with a sulfonamide as a local dressing to wounds that had not responded previously to a sulfonamide alone.⁴ Tsuchiya and his associates^{5, 6, 7} found the combined action of the two substances effective against certain organisms in vitro, particularly staphylococci and *B. coli*. When this idea was applied in vivo to wounds, compound fractures, and infected dermatoses, it met with some

Presented at the Sectional Meeting of the American Federation for Clinical Research, Cornell University, New York, Dec. 4, 1943.
Received for publication, April 24, 1944.

degree of success.^{8, 9} When used in this way externally, urea is thought to increase the solubility of the sulfonamide, to enhance its bacteriostatic action, and to prevent the sulfonamide from caking.

Because of its property to increase sulfonamide solubility,¹⁰ Sobin and his associates gave urea internally in an attempt to reduce the toxic effects of the sulfonamides on the kidneys.¹¹ They administered urea by mouth to rats and considered it to be of some benefit in preventing the renal precipitation of acetylsulfonamide crystals. No toxic effects were encountered.

Such studies of the combined use of an anti-inhibitor, as urea, and the sulfonamides seem important in attempting to broaden our scope of knowledge of the sulfonamide group of drugs.

MATERIAL AND METHODS

At the time this study was undertaken, sulfonamide therapy for gonorrhea in the Army¹² consisted in the administration of 4.0 Gm. of sulfathiazole or sulfadiazine, given in doses of 1.0 Gm. four times daily for five days. The majority responded to this treatment. If it failed, the same course was repeated, or the patient was hospitalized and given 4.0 Gm. of either drug as an initial dose, followed by 1.0 Gm. every four hours, day and night, for five days. If the discharge still persisted the case was labeled, "sulfa-resistant."

Patients were selected for this study who had been sent to the Valley Forge General Hospital as being "sulfa-resistant"; their urethral smears were persistently positive for gonococci. The majority had been given sulfathiazole and had had both the above courses of treatment. Several had had as many as five courses. Treatment was withheld for several days after admission to be certain there was no lag in response to their pre-admission therapy, and to obtain additional data. Besides a careful history and physical examination, such studies included complete blood examination, a second- or third-glass urine specimen for albumin and red blood cells, fasting blood urea nitrogen, and 24-hour urine urea. When all these findings were normal the patient was started on urea in the following mixture:

Urea crystals	325.0 Gm.
Distilled water	450.0 c.c.
Simple syrup q.s. ad	1000.0 c.c.

This mixture was tolerated very well and in the dose of two tablespoons three times a day supplied about 30.0 Gm. of urea daily. This treatment alone was given for three days. On the morning of the third day another fasting blood urea nitrogen determination was made and the collection of a second 24-hour urine for urea content was begun. On the morning of the fourth day urethral smears were examined again to determine whether gonococci were still present. The patient was then begun on a sulfonamide, the same drug and in the same dosage which had failed previously.

The urea and sulfonamide were continued together on the fourth, fifth, and sixth days; then the urea was stopped. If there were no complications as shown by symptoms, blood counts, or urinary findings, the sulfonamide was continued for another three days. The urine was examined daily by the "two-glass test."

If, at the end of treatment, any discharge remained, smears, and in some instances, cultures, were made. The patient was then observed for another three weeks and his progress followed by two-glass tests of the urine and smears. The total treatment as outlined above required nine days, three days on urea alone, three days on urea plus sulfonamide, and three days on sulfonamide alone.

RESULTS OF THERAPY

Forty male patients were treated by this method. Thirty-six were white, four Negro. Thirty-eight were given sulfathiazole, two were given sulfadiazine. All of the patients showed persistence of gonococci after three days on urea alone.

Of the 40 cases, 21, or 52.5 per cent, showed a rather dramatic recovery on the urea and sulfonamide, that is, the urethral discharge ceased abruptly and completely. The two cases treated with sulfadiazine are in this group. We have called these 21 cases successful because there was complete clearing of the two-glass urine specimen, and there was complete absence of symptoms and urethral discharge, with no recurrence over a subsequent three-week period of observation. In the majority of these successful cases the discharge and symptoms usually disappeared on the sixth or seventh day of the treatment, that is, on the third or fourth day that the sulfonamides were administered.

In looking for some causes for failure, the 19 cases that did not respond favorably to the combined treatment were compared in certain respects to the 21 successful cases.

TABLE I

SHOWING RELATIONSHIP BETWEEN TIME INTERVAL FROM APPEARANCE OF DISCHARGE TO INSTITUTION OF ORIGINAL SULFONAMIDE THERAPY, AND THE LATER RESPONSE TO UREA-SULFONAMIDE TREATMENT

NO. OF CASES	DAYS OF URETHRAL DISCHARGE BEFORE ANY THERAPY	THERAPY WITH UREA-SULFONAMIDE	
		SUCCESS	FAILURE
31	Immediate	16	15
2	4	0	2
2	7	2	0
1	11	1	0
1	14	1	0
1	35	0	1
2	60	1	1

There was no apparent relationship between the failure of sulfonamide-urea therapy and the interval between onset of the urethral discharge and institution of the original sulfonamide administration (Table I). Of the total group, 31 had had sulfonamide treatment started immediately upon the appearance of a discharge. Sixteen of these were treated successfully by urea-sulfonamide, 15 failed. Two cases that had gone eleven and fourteen days before any therapy was given were both treated successfully with urea-sulfonamide, but 2 cases that had gone only four days, 1 that had gone thirty-five days, and 1 of 2 cases that had gone eight weeks without treatment were failures.

As shown in Table II, the number of previous attacks of gonorrhea seemed to make no difference in the end results of urea-sulfonamide therapy. Five of the total group had had one previous attack of gonorrhea, 3 had had 2 attacks. It might be thought that previous attacks of the disease would build

degree of success.^{8,9} When used in this way externally, urea is thought to increase the solubility of the sulfonamide, to enhance its bacteriostatic action, and to prevent the sulfonamide from caking.

Because of its property to increase sulfonamide solubility,¹⁰ Sobin and his associates gave urea internally in an attempt to reduce the toxic effects of the sulfonamides on the kidneys.¹¹ They administered urea by mouth to rats and considered it to be of some benefit in preventing the renal precipitation of acetylsulfonamide crystals. No toxic effects were encountered.

Such studies of the combined use of an anti-inhibitor, as urea, and the sulfonamides seem important in attempting to broaden our scope of knowledge of the sulfonamide group of drugs.

MATERIAL AND METHODS

At the time this study was undertaken, sulfonamide therapy for gonorrhea in the Army¹² consisted in the administration of 4.0 Gm. of sulfathiazole or sulfadiazine, given in doses of 1.0 Gm. four times daily for five days. The majority responded to this treatment. If it failed, the same course was repeated, or the patient was hospitalized and given 4.0 Gm. of either drug as an initial dose, followed by 1.0 Gm. every four hours, day and night, for five days. If the discharge still persisted the case was labeled "sulfa-resistant."

Patients were selected for this study who had been sent to the Valley Forge General Hospital as being "sulfa-resistant"; their urethral smears were persistently positive for gonococci. The majority had been given sulfathiazole and had had both the above courses of treatment. Several had had as many as five courses. Treatment was withheld for several days after admission to be certain there was no lag in response to their pre-admission therapy, and to obtain additional data. Besides a careful history and physical examination, such studies included complete blood examination, a second- or third-glass urine specimen for albumin and red blood cells, fasting blood urea nitrogen, and 24-hour urine urea. When all these findings were normal the patient was started on urea in the following mixture:

Urea crystals	325.0 Gm.
Distilled water	450.0 c.c.
Simple syrup q.s. ad	1000.0 c.c.

This mixture was tolerated very well and in the dose of two tablespoons three times a day supplied about 30.0 Gm. of urea daily. This treatment alone was given for three days. On the morning of the third day another fasting blood urea nitrogen determination was made and the collection of a second 24-hour urine for urea content was begun. On the morning of the fourth day urethral smears were examined again to determine whether gonococci were still present. The patient was then begun on a sulfonamide, the same drug and in the same dosage which had failed previously.

The urea and sulfonamide were continued together on the fourth, fifth, and sixth days; then the urea was stopped. If there were no complications as shown by symptoms, blood counts, or urinary findings, the sulfonamide was continued for another three days. The urine was examined daily by the "two-glass test."

to rule out any possible benefit from the bicarbonate. As shown by Fox and Rose¹⁴ and Schmelkes,¹⁵ alkalis may enhance the bacteriostatic action of the sulfonamide. Four patients were given sodium bicarbonate alone, 4 others received it with regular doses of sulfathiazole, and in none was there an effect on the gonorrhea. All 12 patients who had failed on the larger (double) dose schedule of sulfathiazole had been given 2.0 Gm. of sodium bicarbonate every four hours. From this we concluded that the alkali we had administered was no factor in the successful results obtained with urea-sulfonamide treatment.

Of the 19 failures in the series, 8 responded to anterior urethral instillations of silver salts. In this small group we gained the distinct impression that these patients responded much more quickly to the silver salts after having had the urea-sulfonamide treatment, i.e. in four or five days, than had previously similar cases which had been treated with sulfonamides alone. Two patients were given fever therapy with success. At about this time small amounts of penicillin became available to us and the other 9 patients responded dramatically to its administration.

Only 5, or 12.5 per cent, of the 40 patients developed any toxic effects from the sulfonamides. In 4 instances these were mild, including headache, drowsiness, conjunctivitis, and microscopic hematuria. One patient had a severe reaction with fever, malaise, and headache after two days of drug treatment. These patients had all received sulfathiazole. There were no untoward effects from the urea.

LABORATORY STUDIES

Before beginning the urea-sulfonamide therapy, the fasting blood urea nitrogen ranged between 14 and 18 mg. per 100 c.c. The urine urea content averaged 25 to 35 Gm. per 24 hours. After the patient had taken urea for three days the fasting blood urea nitrogen ranged between 16 and 20 mg. per 100 c.c. and the 24-hour urine contained between 30 and 40 Gm. of urea.

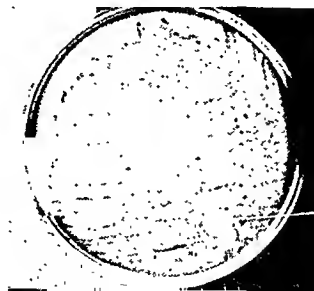
In our laboratory, when patients are treated with sulfathiazole with the army dosage as outlined, the blood level of drug averages between 4 and 7 mg. per 100 c.c. With the urea-sulfonamide treatment, using the same dose of sulfathiazole, the blood levels of drug ranged between 4 and 8 mg. per 100 c.c.

In 14 of the 21 successfully treated cases cultures of urethral strippings were made at weekly intervals for three weeks. All were negative. In any of the failures in which cultures were made, gonococci persisted.

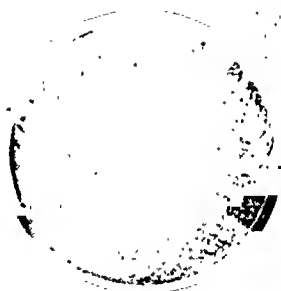
Laboratory confirmation of this clinical experiment was sought. Using the technique of Goodale and his associates,¹⁶ cultures of gonococci from these resistant cases were made on chocolate agar. In addition to a control, chocolate agar plates were used containing 0.1 mg., 0.25 mg., and 0.50 mg. per cent sulfathiazole. Other chocolate agar plates containing 0.5 per cent, 1.0 per cent, and 2.0 per cent urea were implanted with the same strain of organism. A third set of chocolate agar plates containing both chemicals in combinations of the above strength were also implanted. The results are shown in Figs. 1 and 2.

In Fig. 1 it will be noted that gonococci grew out in significant numbers in all strengths of sulfathiazole alone and urea alone. In these cultures we

did not observe the urea-inhibiting effect described by Kirby.¹⁷ Kirby has reported that 2.0 per cent urea, and often as little as 0.2 per cent strength, definitely inhibits the growth of cultures of *B. coli*. In all our cultures the gonococci grew readily on the agar-urea plates, occasionally even better than on the control plate. From the 5 patients treated with urea alone for a week, and the lack of any effect of three days of urea in our 40 patients, we feel that



Chocolate Agar Control



S = 0.1mg%



S = 0.25mg%



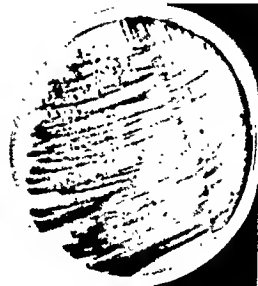
S = 0.5mg%



U = 0.5%



U = 1.0%



U = 2.0%

Fig. 1.—Sulfonamide-resistant gonococci on chocolate agar, chocolate agar with sulfathiazole and with urea. S = Sulfathiazole. U = Urea.

urea alone in the doses used has no bacteriostatic or bacteriolytic effect on gonococci in vitro or in vivo.

Of interest in Fig. 2 is a definite tendency to diminished growth of gonococci in increasing strengths of combined urea and sulfathiazole when compared to the same strengths of either substance alone. The three strengths of sulfathiazole with 2.0 per cent urea showed no growth. This gives the appearance of a potentiating effect.

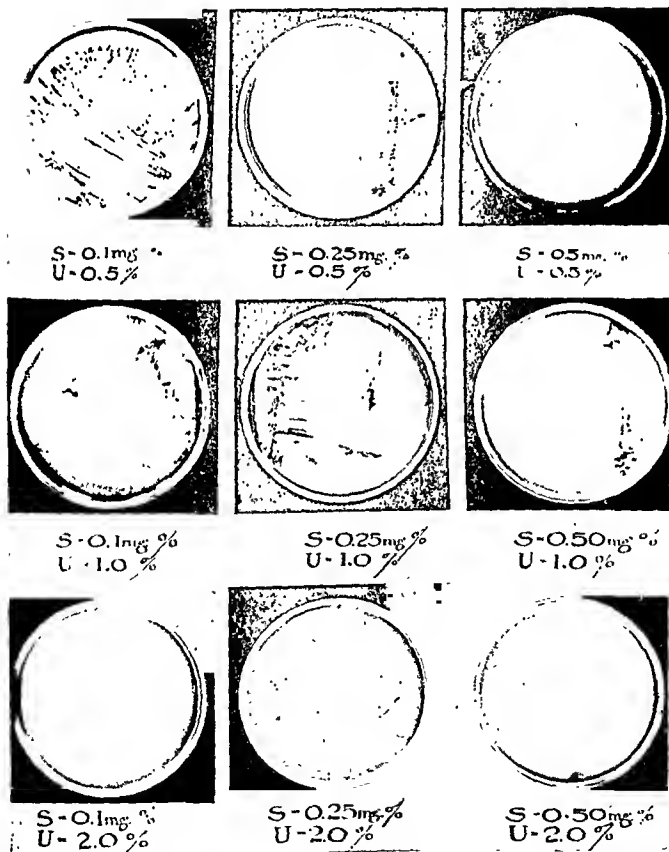


Fig. 2—Sulfonamide-resistant gonococci on chocolate agar with various concentrations of sulfathiazole and urea. S = Sulfathiazole. U = Urea.

8. Strakosch, E. A., and Clark, W. G.: The Beneficial Effect of Urea in Topical Sulfonamide Therapy. I Treatment of Infected Dermatoses. II. Effectiveness of Urea-Sulfonamide combinations in Sulfonamide-Resistant Infections, *Minnesota Med.* 26: 276, 1943.
9. Ilfeld, F. W.: Carbanide Sulfonamide Mixtures; Use in Treatment of Compound Fractures and Traumatic Wounds, *Surg., Gynec. & Obst.* 76: 427, 1943.
10. Sobin, S. S.: Sulfonamide Solubility in Urea, *J. LAB. & CLIN. MED.* 27: 1567, 1942.
11. Sobin, S. S., Aronberg, L. M., and Rolnick, H. C.: The Nature of the Renal Lesion With the Sulfonamides and Its Prevention With Urea, *Am. J. Path.* 19: 211, 1943.
12. Circular Letter, Surgeon General's Office, No. 129, July 22, 1943.
13. Wright, Irving S.: Personal communication.
14. Fox, C. L., Jr., and Rose, H. M.: Ionization of Sulfonamides, *Proc. Soc. Exper. Biol. & Med.* 50: 142, 1942.
15. Schmelkes, F. C., Wyss, O., Marks, H. C., Ludwig, B. J., and Strandkov, F. B.: Mechanism of Sulfonamide Action; Acidic Dissociation and Antibacterial Effect, *Proc. Soc. Exper. Biol. & Med.* 50: 145, 1942.
16. Goodale, W. T., Gould, R. G., Schwab, L., and Winter, V. G.: Laboratory Identification of Sulfonamide Resistant Gonococcal Infections, *J. A. M. A.* 123: 547 (Oct. 30) 1943.
17. Kirby, W. M. M.: In Vitro Action of Urea-Sulfonamide Mixtures, *Proc. Soc. Exper. Biol. & Med.* 53: 109, 1943.

ACUTE AGRANULOCYTOSIS DURING SULFAMERAZINE THERAPY

GRANT O. FAVORITE, M.D., PHILADELPHIA, PA., LEOPOLD REINER, M.D.,
CAMDEN, N. J., AND RUSSELL LONDON, M.D. PHILADELPHIA, PA.

THE general use of the various members of the sulfonamide drugs has been followed by reports of toxic manifestations involving the blood and blood-forming organs. Acute agranulocytosis has been observed in patients to whom sulfanilamide,^{1, 2} sulfapyridine,^{3, 4} sulfathiazole,^{5, 6} sulfadiazine,⁷ and succinyl-sulfathiazole were administered.⁸ The following case report adds sulfamerazine to this list.

REPORT ON CASE

S. M., a white male, 51 years of age, was hospitalized because of a neck injury. On admission, in addition to his injury, he had an acute pharyngitis with a fever of 104.1° F., pulse 120, and respiration 22. His past history was irrelevant. Examination revealed a poorly nourished individual. All motions of the neck were restricted by muscle spasm. The pharynx was injected and ruddy in appearance. Diffuse coarse râles were heard over the tracheal area. The blood pressure was 128 systolic and 78 diastolic. The remainder of the physical examination was negative. Examination of the blood showed the hemoglobin to be 16 Gm. per 100 c.c. of blood, red blood cell count 4,750,000 per c.mm., and the white blood cell count 8,800 per c.mm. Differential showed 76 per cent polymorphonuclears, 22 per cent lymphocytes, and 2 per cent monocytes. The urinalysis was essentially negative.

The patient was given 0.5 Gm. of sulfamerazine twice during the first day and three times daily thereafter for fourteen days. The temperature returned to normal within twenty-four hours, and his general condition appeared normal. Because of the neck injury, a "Queen Anne" plaster cast collar was applied. The following twelve days were uneventful. On the fifteenth day after admission, the patient developed a tracheobronchitis and the dose of sulfamerazine was increased to 1 Gm. three times daily. No other drugs were administered. Symptoms subsided materially after two days, but the temperature fluctuated between normal and 101° F. for the following six days. A blood count at this time revealed hemoglobin, 14 Gm.; red blood cells, 4,350,000; white blood cells, 3,000; polymorphonuclears, 7; and lymphocytes, 93. Because of neutropenia, the drug was discontinued. Forty-two grams were administered during the 23 days.

On the following day the patient's general condition became worse. Respirations were faster. The appearance of a cough, with blood-tinged sputum on one occasion, indicated that there had been an exacerbation of the tracheobronchitis. A roentgenogram of the lungs on this day showed an intensification

From the West Jersey Hospital, Camden, N. J.
Received for publication, May 17, 1944

REFERENCES

1. Long, P. H., Haviland, J. W., Edwards, L. B., and Bliss, E. A.: Toxic Manifestations of Sulfanilamide and Its Derivatives, *J. A. M. A.* **115**: 364, 1940.
2. Dolgopel, V. B., and Hobart, H. B.: Granulocytopenia in Sulfapyridine Therapy, *J. A. M. A.* **113**: 1012, 1939.
3. Kennedy, P. C., and Finland, M.: Fatal Agranulocytosis From Sulfathiazole, *J. A. M. A.* **116**: 295, 1941.
4. Sutliff, W. D., Helpert, M., Griffin, G., and Brown, H.: Sulfonamide Toxicity as a Cause of Death in New York City in 1941, *J. A. M. A.* **121**: 307, 1943.
5. Curry, J. J.: Acute Agranulocytosis Following Sulfadiazine, *J. A. M. A.* **119**: 1502, 1942.
6. Johnson, S. A. M.: Acute Agranulocytosis Due to the Administration of Succinylsulfathiazole, *J. A. M. A.* **122**: 668, 1943.

ISOLATION OF BACTERIUM TULARENSE FROM THE SPUTUM OF AN ATYPICAL CASE OF HUMAN TULAREMIA

HAROLD N. JOHNSON, M.D., MONTGOMERY, ALA.

THE early diagnosis of typhoidal or pneumonic tularemia depends on the isolation of *Bacterium tularensis*.¹ A positive blood culture can be obtained, in some cases, by the use of special media such as blood dextrose cystine agar, but bacteremia has not been demonstrated with regularity except in the terminal stage of fatal cases. There have been few attempts to isolate the organism from the secretions of the respiratory tract, chiefly, no doubt, because of the paucity of sputum and lack of clinical signs of respiratory infection. In the course of an experimental study of tularemia in dogs it was noted that *B. tularensis* exhibited a particular affinity for the mucosa of the upper respiratory tract and in some instances persisted in the nasopharynx after clinical recovery.² Isolation of the organism was accomplished by mouse inoculation and subsequent culture on blood dextrose cystine agar.

A study was therefore made to see whether *B. tularensis* exhibited a similar predilection for the respiratory tract in human infections.

CASE HISTORY

S. L., a twelve-year-old white schoolgirl, was admitted to the Maxwell Field Post Hospital on May 6, 1943, because of persistent fever of unknown etiology. The presenting symptoms were fever and sore throat. She gave a history of having had fever since April 24, 1943. A local physician had prescribed gamune, but the girl had derived no benefit from the drug. Her oral temperature had ranged from 100° to 103.4° F. She did not look sick. Results of physical examination were essentially negative, except that a slight enlargement of the spleen was detected. There was evidence of subacute inflammation of the pharynx, but this did not appear significant. A chest x-ray examination gave negative results. Agglutination tests were run for undulant fever, typhoid, typhus, and tularemia. The blood serum agglutinated *B. tularensis* antigen through a dilution of 1:320. The other agglutination tests were negative.

A review of the girl's history revealed that some children in the neighborhood had found a litter of young wild rabbits on April 18, 1943, and that the girl had played with these rabbits. She had tried to keep the rabbits alive by artificial feeding, but all of them died.

The girl was discharged from the hospital on May 16, 1943. She was kept from having contact with other children but otherwise was allowed ordinary activity. Her temperature was normal except for occasional slight fever in the late afternoon.

METHOD OF STUDY

Owing to the presence of an inflammatory process in the pharynx and the lack of other localizing signs it appeared probable that the throat was the site of the primary lesion. In order to find out whether the patient was still harboring *B. tularensis* and might be a potential source of infection to others, an attempt was made to isolate the organism. The first specimen studied consisted of material obtained from a throat swab taken on May 11, the seventeenth

The studies and observations herein reported were conducted with the support and under the auspices of the International Health Division of The Rockefeller Foundation and the Alabama State Board of Health.

Received for publication, April 7, 1944.

day of illness. This was immersed in 4 c.c. of veal infusion broth and sent to the laboratory of the Alabama State Board of Health, where 0.5 c.c. of the broth suspension was injected intraperitoneally into each of two white mice. The mice were prostrate on the fourth day, and, when killed, showed typical lesions of tularemia in the liver and spleen. A pure culture of *B. tularensis* was obtained on blood dextrose cystine agar (Difeo) from the mouse spleen, and the organism was agglutinated out to full titer with a known positive serum.

As the first specimen proved positive, another specimen was requested. This was obtained on May 19, the twenty-fifth day of illness. It consisted of a small amount of sputum which looked like normal saliva. This sputum was diluted with an equal amount of distilled water and four mice were injected intraperitoneally, each receiving 0.5 c.c. of the suspension. All of the inoculated mice developed tularemia.

A third suspension of sputum was obtained on May 24, the thirtieth day of illness. Four mice were inoculated intraperitoneally with this material as noted above. Again all four mice developed tularemia. A pure culture was obtained on blood cystine agar from mouse spleen, and the identity of the organism was proved by its agglutination to full titer with a known immune serum.

Sputum obtained on May 29th, the thirty-fifth day of illness, was negative by mouse inoculation, as was also the material obtained from a throat swab taken on June 6. On June 24 we received some scrapings from a yellow membranous lesion on the girl's right anterior pharyngeal pillar. The scrapings were placed in broth immediately after they were obtained. The material was triturated and injected intraperitoneally into mice and guinea pigs, but these animals remained well.

EPIDEMIOLOGICAL STUDIES

It was found that nine of the girl's playmates had also handled the young rabbits. Blood specimens were taken from these children on June 24. Agglutination tests performed on the sera were all negative. The blood sera of three dogs from a near-by farm were also tested. One of these dogs gave a positive agglutination with *B. tularensis* antigen in a serum dilution of 1:80.

OBSERVATIONS AND DISCUSSION

It is obvious that the diagnosis of tularemia would not have been made in this case if agglutination tests had not been performed. The illness would have been classed as fever of undetermined origin. The history of contact with rabbits was not elicited until the correct diagnosis had been made. The patient had no primary lesion on either hand, nor was there axillary adenopathy. This suggests that infection took place by way of the mouth or nasopharynx. Cases similar to this one have been reported by Anschuetz³ and Waddell and Birdsong.⁴

It is possible that tularemia could be spread from person to person by inhalation of infected droplets resulting from coughing or sneezing. Although tularemia shows little ability to pass from person to person, should it once achieve this, an alteration of tropism might lead to greater infectiousness by this route. Kennedy⁵ reported a fatal case of pulmonary tularemia in a young

girl with no history of contact with rabbits or exposure to ticks. There are numerous records of cases of tularemia pneumonia without any evident portal of entry. Blackford⁶ recommends testing the sera of all persons with unusual pulmonary lesions for *B. tularensis* agglutinins, since several unsuspected cases of tularemia have been discovered by him in this way.

The diagnosis of tularemia must be considered in cases of cryptogenic fever and atypical pneumonia. Agglutinins do not develop in the blood serum until the second or third week of the disease, the early diagnosis of tularemia, therefore, depends on the isolation of the infecting organism from the blood, from the primary lesion if present, or from the sputum in cases of tularemia pharyngitis and pneumonia. The only satisfactory method at present for proving the existence of the organism in the sputum or nasopharynx is animal inoculation. The mouse appears to be a particularly suitable animal for this purpose. As the sputum is apt to be scanty in cases of tularemia pharyngitis and pneumonia, it will usually be best to collect specimens with a swab. Swabs of both the nasopharynx and throat should be taken, and these should be immersed in a small amount of distilled water or veal infusion broth. The suspension of the material from the swab may then be inoculated intraperitoneally into mice or guinea pigs.

SUMMARY

This report describes the isolation of *B. tularensis* from the sputum of a child with a nonfatal case of tularemia. The patient was a twelve-year-old girl, who was admitted to the hospital because of unexplained fever. *B. tularensis* was isolated by mouse inoculation on three occasions from the seventeenth to the thirtieth day after the onset of the disease. The identity of the organism was proved by culture and cross agglutination tests with known strains of *B. tularensis*. The last two isolations were made after the patient was discharged from the hospital and had resumed normal activity.

The persistence of *B. tularensis* in the respiratory tract indicates that there is a potential danger of contact infection. The white mouse is recommended as a suitable laboratory animal for diagnostic inoculation studies in suspected cases of tularemia.

The author wishes to express his appreciation to Colonel Jacob Brem, M.C., Maxwell Field Post Hospital, Montgomery, Alabama, for making available the specimens used in this study.

REFERENCES

1. Ransmeier, J. C., and Schaub, I. G.: Direct Cultivation of *Bacterium tularensis* from Human Blood Drawn During Life and at Autopsy, *Arch. Int. Med.* 68: 747, 1941.
2. Johnson, H. N.: Natural Occurrence of Tularemia in Dogs Used as a Source of Canine Distemper Virus, *J. LAB. & CLIN. MED.* 29: 906, 1944.
3. Anschuetz, R. R.: Tularemia with Extensive Pharyngitis, *Am. J. Dis. Child.* 62: 150, 1941.
4. Waddell, W. W., Jr., and Birdsong, M.: Tularemia with Local Lesions Confined to the Tonsils, *J. Pediat.* 20: 368, 1942.
5. Kennedy, J. A.: Pulmonary Tularemia, *J. A. M. A.* 118: 781, 1942.
6. Blackford, S. D.: Pulmonary Manifestations in Human Tularemia, *J. A. M. A.* 104: 891, 1935.

NATURAL OCCURRENCE OF TULAREMIA IN DOGS USED AS A SOURCE OF CANINE DISTEMPER VIRUS

HAROLD N. JOHNSON, M.D., MONTGOMERY, ALA.

THE use of a live virus vaccine for the immunization of animals requires especial care in order to avoid spreading other diseases accidentally derived from the animals used as a source of virus. Dogs may be naturally infected with the virus of lymphocytic choriomeningitis,⁶ and this disease may be maintained as an inapparent infection in the course of serial passage of canine distemper virus.¹ The following report describes the isolation of *Bacterium tularensis* from dog spleen tissue used for the production of canine distemper vaccine.

There are accounts, on record, of dogs having developed tularemia in nature.^{2, 3} The diagnosis has depended on the development of an acute illness following contact with wild rabbits and the subsequent demonstration of a high serum agglutination titer for *B. tularensis*. The natural susceptibility of the dog to tularemia has been questioned, because the causative organism has not been isolated from the blood or tissue of a naturally infected dog and properly identified. Tularemia infections have been reported in man following the bite or scratch of cats.⁴ The study of tularemia and its course in experimentally infected dogs, cats, and other animals has revealed some information which may be of value; the pertinent findings are presented herewith.

MATERIALS AND METHODS

In the course of a study of canine rabies it has been necessary to protect the experimental animals from intercurrent infections, notably canine distemper. The usual procedure has been to give one subcutaneous injection of a formalin inactivated dog spleen tissue vaccine, followed four to six weeks later by an injection of active distemper virus.

The specimen from which *B. tularensis* was obtained consisted of a pool of spleen tissue from twenty-five puppies that were killed at the time of maximum febrile response following a simultaneous natural and experimental exposure to canine distemper at a commercial biological laboratory. The puppies were from farms and villages in an area where tularemia is endemic among wild rabbits. Each puppy was given a subcutaneous injection of 1 c.c. of a 20 per cent suspension of spleen tissue from a previous passage of the canine distemper virus. The puppies were then kept in a distemper kennel where there was a high likelihood of superimposed natural exposure. A normal ferret was given a subcutaneous injection of 1 c.c. of a 20 per cent suspension of the dog spleen pool noted above and was sent to this laboratory by Railway Express. The animal was received the following day. An initial febrile reaction developed on the second day after inoculation, and for a period of forty-eight hours the rectal temperature varied from 102.3° to 104.7° F. By the seventh day the tempera-

The studies and observations herein reported were conducted with the support and under the auspices of the International Health Division of The Rockefeller Foundation and the Alabama State Board of Health.

Received for publication, May 11, 1944.

*Lymphocytic choriomeningitis virus has been isolated from commercial canine distemper virus specimens at this laboratory.

ture was normal. There was a second febrile period beginning on the eighth day, reaching a maximum on the eleventh day, when a rectal temperature of 107.9° F. was recorded. The ferret was by this time obviously sick, refusing food and exhibiting a mucopurulent lacrimal and nasal discharge. The ferret was killed on the thirteenth day after inoculation. Tissue was taken for histological study, and the entire spleen was saved as a source of virus.

The course of infection was typical of that produced by canine distemper in ferrets except for the occurrence of a small abscess at the site of inoculation. At the time of autopsy a smear was made from the contents of the abscess and stained by Gram's method. No bacteria could be identified. The gross post-mortem examination was negative except for moderate enlargement of the liver and spleen. The lungs appeared normal. Paraffin sections were prepared from tissue fixed in acetic Zenker's solution and stained with eosin-methylene blue according to the method of Mallory, but modified in this laboratory to secure better staining of the intracytoplasmic inclusion bodies of rabies and canine distemper. Ethyl eosin (alcohol soluble) adjusted to a pH of 4.5 with acetic acid was used for the primary stain, followed by Unna's alkaline methylene blue stain. There were intracytoplasmic inclusion bodies in the epithelial cells of the bile ducts and in the medullary cells of the adrenal glands consistent with those ordinarily found in canine distemper. Sections of the liver, however, presented an unusual picture due to the presence of miliary foci of coagulative necrosis. We were unable to demonstrate bacteria in paraffin sections of the liver stained by the carbolfuchsin-methylene blue technique for acid-fast organisms or by Gram's method for bacteria.

A portion of the spleen from the ferret was triturated in a mortar and diluted to a 10 per cent suspension in distilled water. The suspension was centrifuged, and four mice inoculated intracerebrally with the supernate. We have followed this procedure routinely at the laboratory in order to avoid introducing lymphocytic choriomeningitis into our dog kennels. On the third day after injection all four of the inoculated mice were sick. They exhibited a mucopurulent lacrimal discharge, their backs were humped, and their furs were ruffled. Two were killed seventy-two hours after inoculation, and the other two died the next day. Smears and impressions of the brain tissue showed an acute meningoencephalitis, with a scanty cellular exudate in the meninges. No bacteria could be identified in preparations stained by Gram's method. Cultures made on blood agar and in veal infusion broth, enriched by the addition of whole rabbit blood to 10 per cent, were negative. Preliminary studies showed that the agent was infectious for mice by intranasal, subcutaneous, and intraperitoneal inoculation. *Dogs, guinea pigs, rabbits, hamsters, and baby chicks* were all highly susceptible, and a uniformly fatal infection resulted within three to five days after intracerebral inoculation. A variety of bacteriological media were used in an attempt to cultivate the infectious agent, including blood dextrose cystine agar. No growth was obtained, and therefore the agent was thought to belong to the virus group of diseases. A rather extended study was made before the infectious agent was proved to be *B. tularensis*.

The inoculum for testing the susceptibility of the various species studied was prepared as follows: infected tissue was weighed, ground in a mortar with crystalline aluminum, and diluted to a 10 per cent suspension by weight with

distilled water. This suspension was centrifuged for ten minutes at 2,500 r.p.m., and the supernate was saved as a source of the infectious agent. In the titration studies, serial decimal dilutions of the stock 10 per cent supernate were made by serially transferring 1 c.c. to 9 c.c. of distilled water. For the neutralization tests a pretitrated stock suspension kept at -70° C. was thawed, and serial decimal dilutions were prepared in distilled water containing 10 per cent human serum. The dilutions were so planned that they contained 1, 10, 100, and 1,000 50 per cent MLD's per 0.03 c.c. when diluted with an equal volume of test serum. This mixture was incubated at 37° C. for one hour and then injected intracerebrally into mice.

The agglutination tests were performed according to the method recommended by the National Institute of Health. The end point was taken as the highest dilution of serum giving a 4+ agglutination reaction.

IDENTIFICATION STUDIES

We soon learned that the agent was not related to canine distemper. Dogs and ferrets immune to canine distemper were fully susceptible to infection. Sera known to be fully active in neutralizing the viruses of lymphocytic choriomeningitis, mouse pneumonia, meningopneumonitis, human influenza, canine distemper, and equine encephalomyelitis were tested by Dr. Frank L. Horsfall, Jr. for their power to neutralize the agent in vitro. In no instance was there any evidence of neutralization. We were also unable to demonstrate any evidence of neutralizing substance in the sera of vaccinated dogs and rabbits previously tested and found immune.

It was noted that tiny coccoid bodies could be demonstrated in large numbers in the spleens of infected mice in impressions stained with Giemsa's stain. These bodies were basically intracellular but were also present in large numbers outside of cells, evidently as the result of disintegration of parasitized cells. They could not be demonstrated with certainty in impressions stained by Gram's method. Impressions of the brain of intracerebrally inoculated mice stained by Giemsa's method showed occasional parasitized cells. The bodies were more numerous in impressions of liver tissue but not as abundant as in the spleen. The bodies appeared blue in preparations stained with the Macchiavello stain.

The first proof of the identity of the agent isolated from the dog spleen material was obtained when the blood of dogs convalescent from infection produced by intramuscular inoculation was tested for agglutinins to *B. tularensis*. The blood of three dogs tested three months after inoculation had titers of 1:40, 1:80, and 1:2560 respectively with *B. tularensis* antigen, and no agglutination was obtained with control bloods from normal dogs.

Cultures were again made on blood dextrose cystine agar. Inoculations were made with loopfuls of spleen material from infected mice. These were again negative. No growth was obtained in veal infusion broth enriched with 10 per cent whole rabbit blood, in Brewer's liquid media containing sodium thioglycolate and methylene blue,⁵ or on blood agar plates. The Brewer's media with whole rabbit blood added to a concentration of 10 per cent was also used with negative results. Cultures were tested for infectivity by mouse inoculation after seven to ten days' incubation, but this material failed to reproduce the disease. A batch of blood dextrose cystine agar was then prepared from de-

hydrated media (Difco) as recommended by Ransmeier.⁶ We readily obtained growth of a gram of negative coccobacillus on this media. The colonies had the typical characteristics of *B. tularensis*, and the identity of the organism was determined by animal inoculation and agglutination by a known positive serum.

Though the blood dextrose cystine agar originally used was unsatisfactory for isolating the organism from infected tissue, it was adequate for maintaining an established avirulent laboratory strain of *B. tularensis*.

A culture of the organism isolated at this laboratory was sent to Dr. Edward Francis of the National Institute of Health who reported that the organism had the typical morphology and animal pathogenicity of *B. tularensis*. It was agglutinated out to full titer with a known antitularensis serum.

TULAREMIA IN DOGS

Dogs have been inoculated intradermally, subcutaneously, intramuscularly, intranasally, and intracerebrally with suspensions of infected tissue. A few animals were exposed by feeding tissue infected with *B. tularensis*. In all instances the animals exhibited evidence of a systemic infection. The incubation period and course of the disease varied with the type of exposure.

Intradermal Inoculation—Two 4-month-old dogs were given 0.1 c.c. of a 10 per cent suspension of infected mouse brain into the skin of the upper lip on each side of the nose. Both developed fever forty-eight hours after inoculation. The cervical lymph nodes became enlarged on the third and fourth days, coinciding with the development of pustules at the site of injection. One of these dogs was killed twenty-eight days after exposure, and mice were injected intraperitoneally with a suspension of tissue from the inoculation site and regional lymph nodes. The mice all developed tularemia. Blood taken from this dog on the twenty-eighth day agglutinated *B. tularensis* antigen in a serum dilution of 1:80. The other dog was bled three months after exposure and had a blood serum agglutination titer of 1:20 with *B. tularensis* antigen.

Subcutaneous Inoculation—Seven mature dogs were injected subcutaneously with 0.25 c.c. of a 10 per cent suspension of infected mouse brain. All developed fever within forty-eight hours. The rectal temperature rose from a normal level of 100-102° F. to 103-105°. The animals also developed a watery lacrimal and nasal discharge. The temperature returned to normal from the third to fifth day. A local area of induration developed at the site of injection on the second to fourth day, and progressed to abscess formation. These abscesses opened spontaneously discharging a serosanguineous fluid. From the seventh to fifteenth day, vesiculopapular skin lesions appeared in the axillary and inguinal regions and over the abdomen. The dogs developed a secondary febrile period on the seventh to eighth days which lasted two to three days. At this time they showed a mucopurulent lacrimal and nasal discharge. They were by this time obviously ill. Two of the dogs had fever on occasions as late as twenty days after inoculation, but all recovered.

Puppies were found to be more susceptible than older dogs. Four 3-month-old puppies were given a subcutaneous injection of 0.25 c.c. of a 10 per cent suspension of infected mouse brain. All four of them developed high fever. One of them died on the eleventh day and another on the twelfth day. Autopsies showed focal necrosis of the liver and spleen and extensive pneumonia.

The infecting organism was recovered from the pneumonic lung in both instances by mouse inoculation. A total of twelve puppies, approximately three months of age, were infected by subcutaneous inoculation. They all developed a severe illness, and three died of the infection. The puppies had higher and more protracted fever than older dogs.

Intramuscular Inoculation.—Four dogs, nine to thirty-three months of age, were injected intramuscularly with 0.5 c.c. of a 10 per cent suspension of infected mouse spleen. They ran a course similar to those inoculated subcutaneously, but the illness was more severe and protracted. One of the dogs had a serum agglutination titer of 1:2560 with *B. tularensis* antigen when bled two months after injection. At five months the titer was 1:1280, and at ten months it was 1:320. When bled at two months, the other dogs had titers of 1:40 and 1:80.

Intranasal Inoculation.—Six dogs, twelve to twenty-four months old, were inoculated intranasally with 0.25 c.c. to 0.5 c.c. of a 10 per cent suspension of infected mouse brain. These dogs developed fever three to four days after exposure. One 12-month-old dog had a rectal temperature of 107.5° on the twelfth day, and another of the same age had a temperature of 106° F. on the tenth day. Both of these dogs were killed on the sixteenth day. Autopsies showed focal necrosis of the liver and enlargement of the spleen. The upper respiratory passages were congested and covered with a mucopurulent exudate. There were a few small areas of early pneumonia. The other dogs recovered. One of the dogs was bled one month after exposure. The blood serum had a titer of 1:160 with *B. tularensis* antigen. Two of the dogs were bled six months after exposure. One had a positive agglutination reaction in a dilution of 1:20, while the other was entirely negative.

Two additional dogs were inoculated intranasally with 0.25 c.c. of a 10 per cent suspension of infected mouse brain. Both developed fever, enlarged cervical lymph nodes, and watery lacrimal and nasal discharge three to four days after exposure. On the eighth to tenth day the discharge from the eyes and nose became mucopurulent. Beginning on the fifth day, specimens of nasal secretion were taken by introducing cotton swabs into the nasopharynx. The cotton swabs were then immersed and rotated in 2 c.c. of distilled water. Four mice were given an intraperitoneal injection of 0.25 c.c. of this suspension. Similar specimens were taken at intervals until two negative results were obtained. The nasal secretion from both dogs was positive for *B. tularensis* by mouse inoculation on the fifth day. Positive specimens were obtained from one dog through the twenty-fifth day and from the other through the twelfth day. These dogs were twelve and twenty-four months old respectively. The older dog carried the organism for the longer period, and appeared normal for ten days preceding the last instance of recovery of the organism. This dog, one month after inoculation, had a serum agglutination titer of 1:160 with *B. tularensis* antigen.

Intracerebral Inoculation.—Four dogs were injected intracerebrally with 0.25 c.c. of a 10 per cent suspension of infected mouse brain. All developed high fever twenty-four hours after injection. Two died on the second day. The other two were dead by the fourth day.

Feeding Infected Tissue.—One 8-month-old dog was fed three mice infected with tularemia. The dog became sick forty-eight hours later. The temperature rose to 104.4° and the eyes and nose showed a mucopurulent discharge. The animal was obviously ill. After an acute illness of five days it gradually improved. Blood was taken daily for mouse inoculation and for culture on blood dextrose cystine agar. We failed to isolate *B. tularensis* from the blood. A few specimens of the nasal secretion were taken by the nasopharyngeal swab method. *B. tularensis* was isolated from the nasopharynx as late as the eighth day after the dog had eaten the infected mice. The isolation was checked by agglutination tests. Blood taken on the tenth day after exposure gave a 4+ agglutination reaction with *B. tularensis* antigen in a dilution of 1:80. The titer rose to 1:640 on the fifteenth day. By the end of three months the titer had dropped to 1:80. Two normal dogs kept in the same pen with this dog failed to develop fever or other evidence of infection. Neither of these dogs developed agglutinins to *B. tularensis*.

Pathology.—The dogs that died or were killed during the secondary febrile period all showed small areas of focal necrosis in the liver and enlargement of the spleen. In several instances the spleen also showed pin-point size areas of focal necrosis. There was enlargement of the lymph nodes related to the site of exposure, and these also contained areas of focal necrosis. The lungs showed slight to extensive pneumonia. Paraffin sections of pneumonic lung stained by the Wolbach modification of Gram's stain revealed enormous numbers of bacteria consistent in appearance with *B. tularensis*. Some alveoli were completely filled with tiny coccoid organisms. Small clumps of bacteria were also found in the sinusoids of the liver. This explains the occurrence of areas of focal necrosis in all parts of the liver lobule. Bacteria could not be demonstrated in the areas of focal necrosis. These foci presented a coagulative type of necrosis with general loss of cell detail.

Despite the rapid and fatal course following intracerebral inoculation, there was no gross evidence of meningitis other than marked vasocongestion. It was usually difficult to find the site of injection. Microscopic examination revealed a scanty inflammatory exudate in the meninges composed primarily of polymorphonuclear cells. There was no deposition of fibrin.

Tissue Infectivity.—Various tissues taken from dogs that died or were killed during the second febrile period were tested for infectivity by mouse inoculation. The infecting organism was recovered from the inoculation site, regional lymph nodes, and spleen. The liver was not tested because it was obvious from the gross appearance that it would be positive. The submaxillary gland did not prove infectious by mouse inoculation.

TULAREMIA IN CATS

Subcutaneous Inoculation.—Two young cats were inoculated subcutaneously with 1 c.c. of a 10 per cent suspension of the ferret spleen material. They developed fever of 104.6° to 106.6° F. forty-eight hours after inoculation. Fever subsided in from forty-eight to seventy-two hours, but the animals appeared sick for two weeks after injection. When reinoculated one month later they exhibited no fever or symptoms of infection. Two young cats were given a subcutaneous injection of 0.25 c.c. of a 10 per cent suspension of infected mouse brain and

were killed on the fourteenth day after inoculation. There were several lobular areas of pneumonia, and the infecting agent was recovered from the lung tissue by mouse inoculation.

Intranasal Inoculation.—Two young cats given 0.1 c.c. of a 10 per cent suspension of infected mouse brain intranasally were killed on the fourteenth day after infection. These also had lobular areas of pneumonia, and the infecting agent was recovered from the lung tissue by mouse inoculation. The cats killed on the fourteenth day after inoculation all showed focal necrosis of the liver and enlargement of the spleen.

Intracerebral Inoculation.—Two adult cats were given 0.1 c.c. of a 10 per cent suspension of infected mouse brain by intracerebral inoculation. Both developed high fever and incoordination twenty-four to forty-eight hours after injection. One died on the eighth day but the other recovered. The cat that recovered was very sick for a period of five days. It was hardly able to get up, a mucopurulent secretion exuded from the eyes and nose, and it appeared apathetic and sleepy.

TULAREMIA IN MICE

Brain tissue from mice infected by intracerebral inoculation was used as a source of the infecting agent for most of the susceptibility experiments, although the spleen contained a somewhat higher concentration of organisms per gram of tissue. Titrations of infected brain gave a titer of at least 0.03 c.c. times 10^{-8} when inoculated either intracerebrally or intranasally. Infected lung tissue had an equally high titer. Owing to the high infectivity by intranasal inoculation, it seemed probable that the disease could be transmitted in mice by contact infection. But when on two occasions groups of normal mice were put into the cage with inoculated mice, the normal mice did not develop the disease; yet all of them were found to be fully susceptible when subsequently tested. An intranasal passage study was initiated from the ferret spleen material. The mice consistently developed extensive pneumonia. Another intranasal passage study was derived from a strain that had been through fifteen intracerebral passages. This strain failed to produce significant pulmonary lesions even after ten serial passages by intranasal inoculation. Despite the absence of appreciable pulmonary lesions, a fatal infection ensued within four days after inoculation. In both passage studies the lung tissue used for the inoculum was taken from mice killed seventy-two hours after injection. Mice were also tested for their susceptibility to infection through the skin. We were unable to infect the mice when a swab of cotton dipped in a 10 per cent suspension of infected tissue was rubbed on the skin; however, if a light needle scratch preceded the application, the mice often developed tularemia. Mice infected by various methods of inoculation consistently developed a *B. tularensis* bacteremia.

TULAREMIA IN BABY CHICKS

Twelve 1-day-old Rhode Island Red chicks were injected intracerebrally with 0.03 c.c. of a 10 per cent suspension of the ferret spleen material. Two were prostrate on the third day. All were dead by the seventh day after inoculation. Groups of six chicks were inoculated with serial tenfold dilutions of

infected chick brain made up in distilled water. All of the chicks inoculated with the 10^{-4} dilution were dead by the fifth day, and 2 of the 6-chick groups inoculated with the 10^{-5} dilution developed tularemia.

TULAREMIA IN OTHER LABORATORY ANIMALS

Guinea pigs inoculated intracerebrally or intraperitoneally with suspensions of infected tissue were usually dead within four days. Normal guinea pigs failed to contract infection by contact with experimentally infected guinea pigs. Hamsters inoculated intracerebrally or intraperitoneally usually died on the third day after injection. Rabbits showed the same incubation period as guinea pigs and died regularly within four days after intracerebral or intraperitoneal inoculation. Ferrets inoculated intranasally with a 10 per cent suspension of infected mouse-lung tissue developed fever in about twenty-four hours, and were usually prostrate on the sixth day after injection.

CULTIVATION OF *B. TULARENSE* IN TISSUE CULTURE

Bacterium tularense multiplied readily in a medium of minced 9-day-old chick embryo tissue and serum Tyrode solution. Transfers were made after three to four days' incubation. The organism remained highly virulent for mice through thirty-one serial subcultures.

EFFECT OF PHYSICAL AND CHEMICAL AGENTS ON *BACTERIUM TULARENSE*

In the course of the tissue-culture experiments we occasionally inoculated flasks containing 4 c.c. of human serum Tyrode solution to see whether the organism would remain alive without the presence of chick embryo tissue. Material from these flasks did not prove infectious for mice after four days' incubation at 37° C.

Infected brain stored frozen at -25° C. retained its infectivity for several months as did also 10 per cent suspensions of infected tissue when they were tubed, sealed, and stored at -70° C. Repeated freezing and thawing of suspensions of infected tissue rendered the material avirulent.

Since dogs developed a fatal infection following intracerebral inoculation, we tested the effect of sulfadiazine in preventing a fatal outcome. Four dogs inoculated intracerebrally with 0.5 c.c. of a 1:1000 dilution of infected mouse brain were given 3 Gm. of sulfadiazine on the day of inoculation and then 1.5 Gm. daily. In this dosage the drug failed to show any inhibitory effect on the course of the disease. Four control dogs were tested in parallel with the treated dogs.

Various dilutions of infected tissue were passed through filters designed to withhold bacteria. The Seitz serum sterilizing filter pad was impervious to the organism. The infecting agent passed through the Berkefeld V grade filter quite readily, but was withheld by the Berkefeld N and W filters.

DISCUSSION

In view of the possible contamination of canine-distemper-virus material with viruses or bacteria not revealed by ordinary sterility tests, such products should not be released for sale until each lot of virus has been tested by animal inoculation and by culture on **blood dextrose cystine agar**. Guinea pigs or mice

VI AGGLUTINATIVE PROPERTIES FOR BACTERIUM TYPHOSUM DEMONSTRATED FOLLOWING INFECTION WITH MALARIA PARASITES

MARION B. COLEMAN, B.S., ALBANY, N. Y.

SINCE the original report from this laboratory on the occurrence of Vi agglutination with *Bacterium typhosum* in sera from patients having therapeutic malaria,¹ specimens have been examined from those having clinical malaria and also from patients having neurosyphilis and treated by intravenous injection of typhoid vaccine. The only report found in the literature of the demonstration of Vi agglutination in a significant number of sera from individuals having infections with agents other than typhoid bacilli is that of Bensted.² He observed such reactions in specimens from six patients having tropical typhus fever, who had previously been inoculated subcutaneously with heat-killed typhoid vaccine. He obtained no Vi agglutination in sera from forty-four other similarly inoculated patients who had tonsillitis, bronchial catarrh, or tuberculosis. He demonstrated transient Vi agglutinative properties within a few days after subcutaneous administration of heat-killed phenolized typhoid vaccine, and suggested that latent or residual Vi agglutinative properties thus induced may subsequently be stimulated by other agents. Felix, Rainsford, and Stokes³ and also Climie⁴ demonstrated Vi agglutination in sera of individuals inoculated subcutaneously with alcohol-killed typhoid vaccine, but in sera from only a few when the vaccine was heat-killed and preserved with phenol. When heat-killed vaccine was administered intravenously, however, Climie observed Vi agglutination in a high percentage of cases.

The association of Vi agglutination with infection with malaria parasites was first suggested, in this laboratory, by results obtained with sera submitted in 1942 from inmates of Sing Sing Prison to be tested for floccular and granular agglutination with typhoid bacilli. As a matter of interest, the sera were also tested for Vi agglutination and, surprisingly, this occurred in relatively high dilutions of the specimens from three individuals who, it was learned later, had received subcutaneous injections of heat-killed typhoid vaccine preceding malarial therapy. A systematic study was then undertaken of specimens from this group of patients, using sera remaining after tests for evidence of syphilis had been completed.

The technic outlined in a previous publication⁵ was followed, and a pure Vi strain of *Bact. typhosum* isolated in this laboratory (No. 37136) was used, in addition to that designated by Bhatnagar⁶ as "Vi I" (No. 39121). No significant difference was observed in the degree of reaction with the two strains. (Since the completion of this study, strain No. 37136 has become

From the Division of Laboratories and Research, New York State Department of Health, Albany.

Presented at the meeting of the Eastern New York Branch of the American Society of Bacteriologists, Albany, April 21, 1944.

Received for publication, April 17, 1944.

unsatisfactory for demonstrating Vi agglutination.) Tests for floccular and granular agglutination were performed as outlined in the Standard Methods⁷ of this Division.

SERA FROM PATIENTS HAVING THERAPEUTIC MALARIA

Sera from thirty-four patients with neurosyphilis or latent syphilis who had been subjected to malarial therapy were studied. Definite Vi agglutination occurred in specimens from twenty-three, and partial or questionable reactions, in those from two. The failure to demonstrate Vi agglutination in sera from the remaining nine patients does not prove that these properties may not have been present at some time. Only one specimen was tested from each of six of the latter; blood examined from the other three was collected either within three weeks or not until four months or more after malarial therapy. Agglutinative properties may have been present in the intervening period since the examination of series of sera from ten other neurosyphilitic individuals indicates that reactions may not be demonstrated until the fifth or sixth week and may not persist for longer than three months after therapy. The peak usually occurred between the third and the fifth week.

Eight of these patients whose sera contained Vi agglutinative properties were said to have had typhoid vaccine: four, subcutaneously within a month; one, subcutaneously twenty-four years previously; and three, intravenously ten days, eighteen months, and twenty months, respectively, before the specimens were collected. On the other hand, fifteen whose sera reacted were reported not to have had typhoid vaccine after admission to Sing Sing, and to have stated that they had not had it previously. Significant floccular agglutination with typhoid bacilli occurred in sera from only one of the latter, suggesting that he may have had vaccine but had forgotten or failed to comprehend the meaning of the inoculation.

It is of particular interest that two attempts to infect one patient with malaria parasites were reported unsuccessful; yet serum collected six weeks later had a Vi agglutination titer of 1:20.

As a control, tests for Vi agglutination were performed with 103 sera from ninety-six inmates of Sing Sing Prison who had received no malarial therapy. Definite reactions were obtained in specimens from only two, in a 1:40 dilution of one and in a 1:10 dilution of the other. Partial reactions of questionable significance occurred in those from seven. The data indicated that typhoid vaccine had not been given to any of the nine. Blood specimens were secured a year later from the two whose sera gave definite reactions, and a titer of 1:40 was again obtained with one but no significant agglutination with the other. Series of three fecal specimens collected at three-day intervals from both individuals were examined for typhoid bacilli; none was found. This incidence of unexplained reactions is not unusual.⁸

SERA FROM PATIENTS HAVING CLINICAL MALARIA

In view of the incidence of Vi agglutination in sera from patients undergoing malarial therapy, specimens were obtained from twenty-eight men in the armed forces who had clinical malaria. Vi agglutinative properties were dem-

VI AGGLUTINATIVE PROPERTIES FOR BACTERIUM TYPHOSUM DEMONSTRATED FOLLOWING INFECTION WITH MALARIA PARASITES

MARION B. COLEMAN, B.S., ALBANY, N. Y.

SINCE the original report from this laboratory on the occurrence of Vi agglutination with *Bacterium typhosum* in sera from patients having therapeutic malaria,¹ specimens have been examined from those having clinical malaria and also from patients having neurosyphilis and treated by intravenous injection of typhoid vaccine. The only report found in the literature of the demonstration of Vi agglutination in a significant number of sera from individuals having infections with agents other than typhoid bacilli is that of Bensted.² He observed such reactions in specimens from six patients having tropical typhus fever, who had previously been inoculated subcutaneously with heat-killed typhoid vaccine. He obtained no Vi agglutination in sera from forty-four other similarly inoculated patients who had tonsillitis, bronchial catarrh, or tuberculosis. He demonstrated transient Vi agglutinative properties within a few days after subcutaneous administration of heat-killed phenolized typhoid vaccine, and suggested that latent or residual Vi agglutinative properties thus induced may subsequently be stimulated by other agents. Felix, Rainsford, and Stokes³ and also Climie⁴ demonstrated Vi agglutination in sera of individuals inoculated subcutaneously with alcohol-killed typhoid vaccine, but in sera from only a few when the vaccine was heat-killed and preserved with phenol. When heat-killed vaccine was administered intravenously, however, Climie observed Vi agglutination in a high percentage of cases.

The association of Vi agglutination with infection with malaria parasites was first suggested, in this laboratory, by results obtained with sera submitted in 1942 from inmates of Sing Sing Prison to be tested for floccular and granular agglutination with typhoid bacilli. As a matter of interest, the sera were also tested for Vi agglutination and, surprisingly, this occurred in relatively high dilutions of the specimens from three individuals who, it was learned later, had received subcutaneous injections of heat-killed typhoid vaccine preceding malarial therapy. A systematic study was then undertaken of specimens from this group of patients, using sera remaining after tests for evidence of syphilis had been completed.

The technic outlined in a previous publication⁵ was followed, and a pure Vi strain of *Bact. typhosum* isolated in this laboratory (No. 37136) was used, in addition to that designated by Bhatnagar⁶ as "Vi I" (No. 39121). No significant difference was observed in the degree of reaction with the two strains. (Since the completion of this study, strain No. 37136 has become

From the Division of Laboratories and Research, New York State Department of Health, Albany.

Presented at the meeting of the Eastern New York Branch of the American Society of Bacteriologists, Albany, April 21, 1944.

Received for publication, April 17, 1944.

SUMMARY

Vi agglutinative properties were demonstrated with typhoid bacilli in significant dilutions of sera from (1) twenty-three of thirty-four patients having neurosyphilis or latent syphilis and who were receiving malarial therapy; (2) two of ninety-six individuals who had received no malarial therapy; (3) eleven of twenty-eight patients with clinical malaria; and (4) fourteen of thirty-one neurosyphilitic patients who had been treated by intravenous injection of typhoid vaccine.

Two hypotheses are offered: (1) that malaria parasites possess Vi antigen and (2) that latent agglutinative properties are stimulated.

These observations should be considered in evaluating Vi agglutination. A continuation of the study should include the examination of various species of bacteria for Vi antigen and the determination of factors that may stimulate latent Vi agglutinative properties.

Grateful acknowledgment is made of the cooperation of Colonel D. M. Kuhns, Director of the Fourth Service Command Laboratory, Ft. McPherson, Georgia, who arranged for the submission of blood specimens from men in the armed forces, and of Dr. B. I. Kaplan and Dr. I. J. Brightman, Ossining, who furnished the data regarding the patients with neurosyphilis or latent syphilis treated by malarial therapy.

REFERENCES

1. Division of Laboratories and Research, New York State Department of Health, Albany, Annual Report, 1942, p. 51-52.
2. Bensted, H. J.: *Bacterium Typhosum*. The Development of Vi-Antigen and Vi-Antibody, J. Roy. Army M. Corps 74: 19, 1940.
3. Felix, A., Rainsford, S. G., and Stokes, E. J.: Antibody Response and Systemic Reactions After Inoculation of a New Type of T.A.B.C. Vaccine, Brit. M. J. 1: 435, 1941.
4. Climic, H.: Immunization Against Typhoid and Paratyphoid With Alcohol-Killed, Alcohol-Preserved and Heat-Killed Phenol-Preserved Vaccine, J. Hyg. 42: 411, 1942.
5. Coleman, M. B.: Experience With the Test for Vi Agglutinative Properties for *Escherichia Typhosa*, Am. J. Pub. Health 32: 843, 1942.
6. Bhatnagar, S. S., Speechly, C. G. J., and Singh, M.: A Vi Variant of *Salmonella Typhi* and Its Application to the Serology of Typhoid Fever, J. Hyg. 38: 663, 1938.
7. Wadsworth, A. B.: Standard Methods of the Division of Laboratories and Research, New York State Department of Health, Baltimore, 1939, Ed. 2, Williams and Wilkins Company, p. 178.

STUDIES ON THE ABSORPTION OF THE SULFONAMIDES FROM THE GASTROINTESTINAL TRACT OF ALBINO RATS

ELMER H. LOUGHLIN, M.D., RICHARD H. BENNETT, M.D.,
MARY E. FLANAGAN, B.S., AND SAMUEL H. SPITZ, M.D.
BROOKLYN, N. Y.

MANY reports on the pharmacology of the sulfonamides in human beings and animals have been published.¹ In these studies, with few exceptions,² observations on the absorption of the sulfonamides as determined by blood levels obtained were seldom made before one hour after their administration to human beings by mouth or by stomach.

Recently, we reported the results of investigations in human beings³ in which we observed the blood levels reached by various sulfonamides during a three-hour period after their administration by mouth. We found that (1) sulfapyridine, sulfathiazole, and sulfadiazine, as well as their sodium salts, were quickly absorbed from the gastrointestinal tract, as evidenced by their rapid appearance in the blood, frequently as early as five minutes after peroral administration; (2) the absorption of sulfapyridine, sulfathiazole, and sulfadiazine was favored by the simultaneous administration of equivalent amounts of sodium bicarbonate; (3) the absorption of the sodium salts, as demonstrated by blood levels attained, was more rapid and greater than that obtained when sulfapyridine, sulfathiazole, or sulfadiazine were given alone or in combination with sodium bicarbonate; (4) conjugated forms of the sulfonamides appeared in the blood as early as five minutes after administration, in some instances being the only forms present; and (5) conjugation of the sulfonamides was greater when the sodium salts were administered than when sulfapyridine, sulfathiazole, and sulfadiazine with bicarbonate of soda were given.

It has been the opinion of some workers in the field of chemotherapy⁴ that absorption of the sulfonamide compounds occurred principally from the small intestine. This concept was based on investigations made after the administration of sulfapyridine by stomach tube to dogs in which the pylorus had been ligated; insignificant amounts of the sulfonamide were found to be absorbed from the stomach. Similar doses administered into the small intestine produced significantly higher blood levels. This study was confined to the levels of free drug in the blood, since conjugation of the sulfonamides was found to occur only slightly in dogs.

In view of our studies showing the rapid absorption of the sulfonamides as indicated by their early appearance in the blood after peroral administration in human beings, we felt that, despite reports to the contrary,⁴ significant quantities of these drugs could be absorbed from the stomach. The purpose of the

From the Department of Internal Medicine, Long Island College of Medicine, Brooklyn, N. Y.
This work was made possible by a grant from the Winthrop Chemical Company, and the Dr. Frank E. West Fund, established by Miss Elizabeth Frothingham, and the John C. Warren Memorial Fund, established by Dr. Luther F. Warren, in memory of his son.

Received for publication, May 10, 1944

present study is to investigate in albino rats the absorption of the sulfonamides from the stomach, as well as to compare these results with those from the stomach and small intestine together.

METHODS AND MATERIALS

The animals used were healthy albino rats, each weighing from 300 to 350 grams. The animals in one group were anesthetized lightly with ether. The abdominal wall was incised, the stomach exposed, the pylorus occluded by a black silk ligature, and the abdomen closed with metal skin clips. Administration of the sulfonamides by gavage was accomplished, and the animals were permitted to return to consciousness within five minutes after completion of the operative procedure.

A second group of animals, in which pyloric occlusion was not done, were given the sulfonamides also by gavage.

Sulfapyridine, sodium sulfapyridine, sulfathiazole, sodium sulfathiazole, sulfadiazine, and sodium sulfadiazine were administered in doses of $\frac{1}{3}$ Gm. of the sulfonamide per kilogram of body weight. This dosage was chosen arbitrarily, the amount of the drug being proportionately the same as that which Long in studies on sulfonamide absorption, administered to mice.⁵ It also was the daily amount of sulfonamide administered by us in the treatment of pneumococcal pneumonia in rats.⁶

Samples of blood for determination of its sulfonamide content were obtained by cardiac puncture five minutes after administration of the drug, and then at intervals of five and ten minutes during the first half-hour and second half-hour periods, respectively. From six animals with pyloric occlusion, to each of which was administered a different sulfonamide, specimens of blood were obtained at intervals of one minute during the first five minutes after administration.

Determinations of free and conjugated sulfonamides in the blood were made according to the procedure of Bratton and Marshall.⁷ A Klett-Summerson photoelectric colorimeter with a No. 54 filter was used.

RESULTS

Sulfapyridine (Charts 1 and 2).—Small amounts of free sulfapyridine (0.072, 1.17, 1.36, and 2.29 mg. per 100 c.c.), were found in the blood in each of four rats with pyloric occlusion five minutes after sulfapyridine had been given. In each of three animals without pyloric occlusion, levels of 1.6, 6.5, and 7.6 mg. per 100 c.c. of the free drug were found.

In three of the four animals with pyloric occlusion both the free and conjugated forms were found in the blood at five minutes after administration, while in three rats without pyloric occlusion the conjugated form was found in one.

In the animals without pyloric occlusion, it was found that the blood levels in two of the three were higher throughout the hour period than in those with pyloric occlusion. In a third animal without pyloric occlusion, which survived only twenty-five minutes, the levels of free drug in the blood were similar to

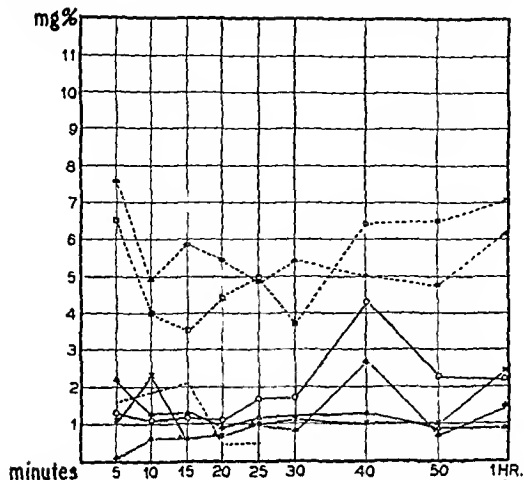


Chart 1.—Free blood levels of sulfapyridine after a single dose of $\frac{1}{2}$ Gm. per kilogram of sulfapyridine administered by gavage.

--- Without pyloric occlusion.
 — With pyloric occlusion.

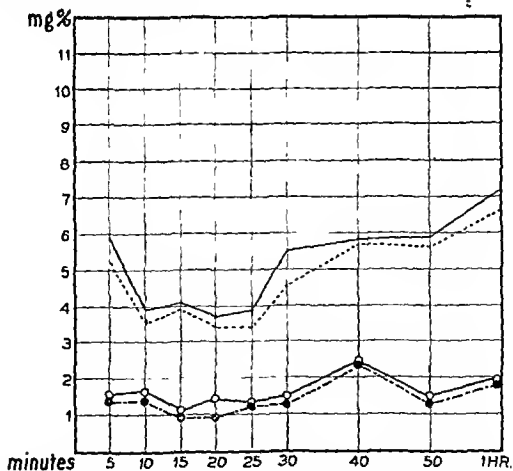


Chart 2.—Average free and total blood levels of sulfapyridine after a single dose of $\frac{1}{2}$ Gm. per kilogram of sulfapyridine administered by gavage.

--- Total Without pyloric occlusion.
 --- Free Without pyloric occlusion.
 — Total With pyloric occlusion.
 — Free With pyloric occlusion.

those in the animals with pyloric occlusion. The degree of conjugation in each, however, was approximately the same.

In two animals with pyloric occlusion to which sulfapyridine had been administered, determinations of the blood sulfonamide were made at one-minute intervals during the first five-minute period. In one animal, free levels of 12.0, 7.7, 7.6, 5.76, and 4.65 mg. per 100 e.e., respectively, were obtained at the first,

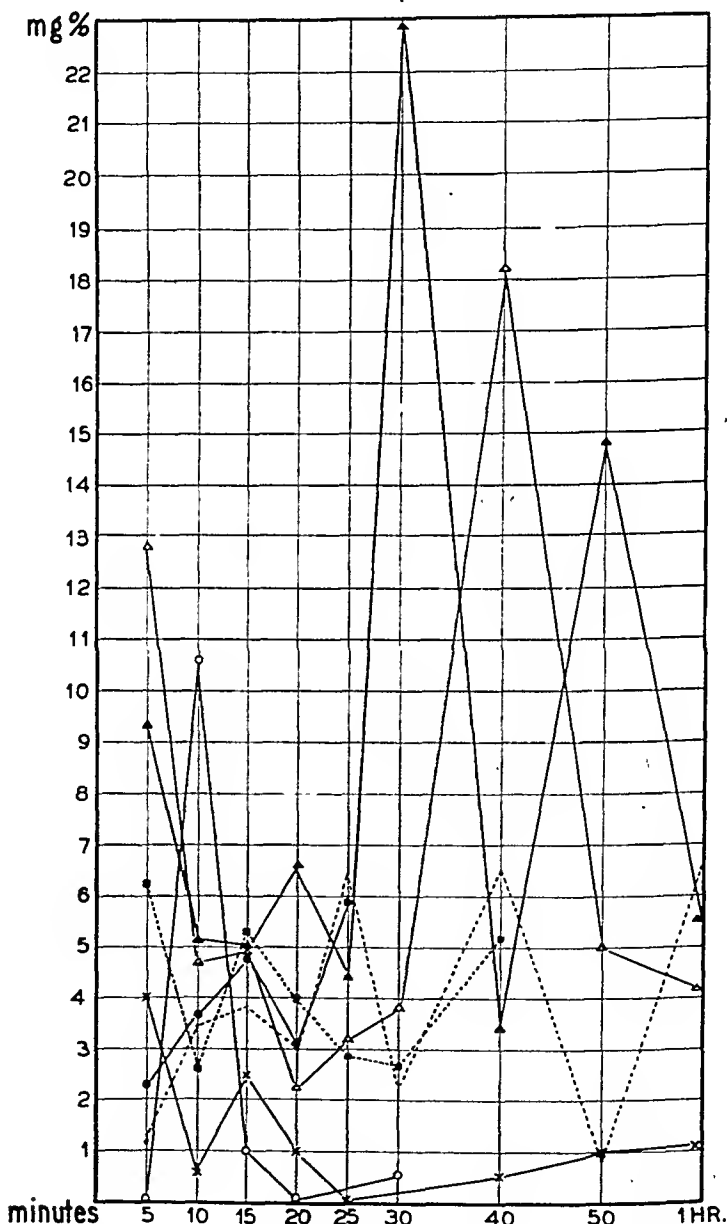


Chart 3.—Free blood levels of sodium sulfapyridine after a single dose of 16 Gm. per kilogram of sodium sulfapyridine administered by gavage.

— Without pyloric occlusion.
— With pyloric occlusion.

second, third, fourth, and fifth minutes. Total levels of the drug in this animal were respectively 12.0, 8.0, 7.6, 5.97, and 4.97 mg. per 100 c.c. A second animal similarly treated had free blood levels of 6.13, 5.33, 3.4, and 2.0 mg. per 100 c.c., and total blood levels of 8.26, 6.93, 4.53, and 4.13 mg. per 100 c.c. at one, two, three, and five minutes.*

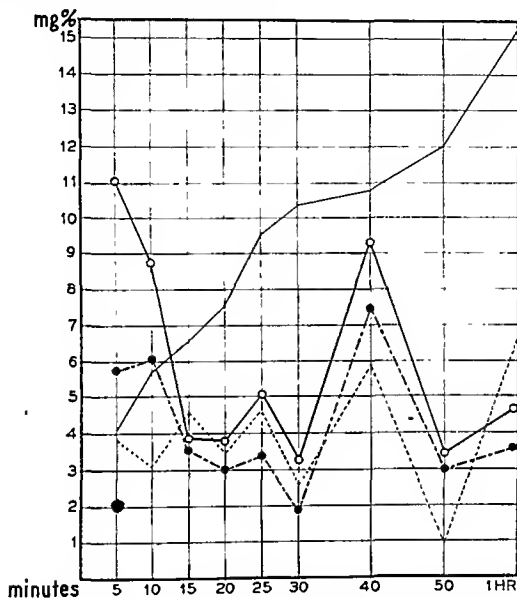


Chart 4.—Average free and total blood levels of sodium sulfapyridine administered by gavage.

--- Total Without pyloric occlusion
 — Free
 o—o Total With pyloric occlusion.
 •—• Free

Sodium Sulfapyridine (Charts 3 and 4).—Following the administration of sodium sulfapyridine, greater quantities of sulfonamide appeared in the blood of each group of animals at five minutes than when sulfapyridine was given. In the rats with pyloric occlusion, the free levels of sodium sulfapyridine in the blood were 0.1, 2.3, 4.0, 9.3, and 12.9 mg. per 100 c.c. and without pyloric occlusion the free levels were 1.4 and 6.3 mg. per 100 c.c.

In three of five animals with pyloric occlusion to which sodium sulfapyridine had been administered, the conjugated as well as the free form was present at five minutes. In a fourth rat the conjugated form alone was present. In two rats without pyloric occlusion, one had conjugated as well as free sodium sulfapyridine in the blood.

*Blood could not be obtained at the fourth minute.

In one animal with pyloric occlusion to which sodium sulfapyridine had been administered, blood sulfonamide determinations were made at minute intervals during the first five-minute period. The free levels obtained were 4.4 and 8.9 mg. per 100 c.c. at one and two minutes, respectively. Traces were found at three and four minutes, while at five minutes, the free form had disappeared entirely, though the conjugated form was present. Conjugated sulfapyridine which appeared as early as one minute after administration, was absent at the second minute, reappeared at the third, and was present at the fourth and fifth minutes.

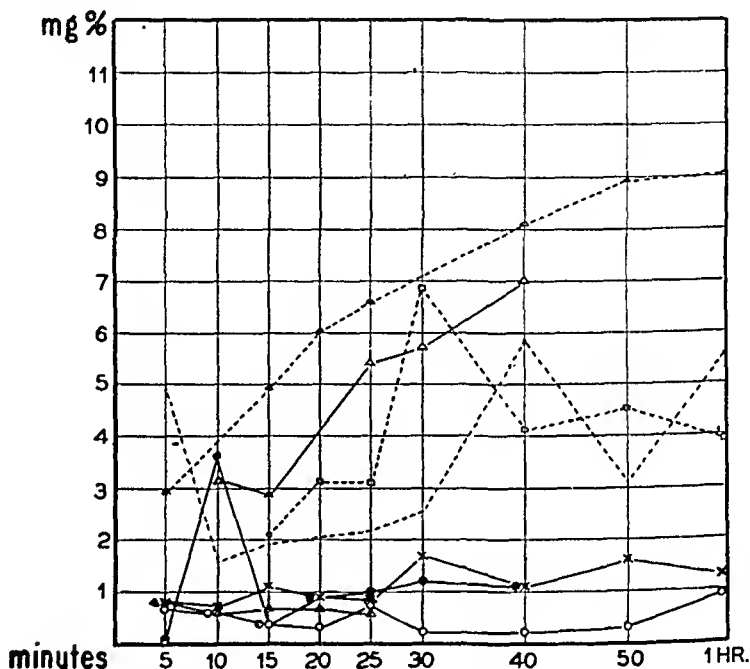


Chart 5.—Free blood levels of sulfathiazole after a single dose of $\frac{1}{2}$ Gm. per kilogram of sulfathiazole administered by gavage.

--- Without pyloric occlusion.
 — With pyloric occlusion.

In the rats receiving sodium sulfapyridine, the free blood levels in those with pyloric occlusion were frequently higher during the hour period than in those without pyloric occlusion. In both groups wide fluctuations occurred, being more pronounced in those with pyloric occlusion. Whereas, the average total level generally ranged close to the free level in those animals whose pylorus was occluded, in those without occlusion the average degree of conjugation progressively increased throughout the entire hour.

Sulfathiazole (Charts 5 and 6).—Free sulfathiazole in small amounts (0.84, 0.84, 0.78, and 0.12 mg. per 100 c.c.), was found in the blood in each of four animals with pyloric occlusion five minutes after the administration of sulfathiazole,* while, in each of two animals without pyloric occlusion, significantly larger quantities (2.9 and 4.8 mg. per 100 c.c.) were present.

*Blood could not be obtained from a fifth rat at five minutes.

In all four rats with pyloric occlusion, conjugated sulfathiazole was present in addition to the free form at five minutes, and one of the two rats without pyloric occlusion had both conjugated and free drug in the blood.

In one rat with pyloric occlusion, determinations for the sulfonamide in the blood at intervals of one minute for the first five minutes were made. Free blood levels in this animal were 0.84, 0.72, 1.15, and 0.84 mg. per 100 c.c. respectively at one, two, three, and five minutes after administration. No specimens of the blood were procured at the fourth minute. Total blood levels, revealing the presence of conjugated sulfathiazole were 1.2, 0.78, and 1.2 mg. per 100 c.c., respectively, at one, two, and five minutes. No determination for conjugated drug was made at three minutes.

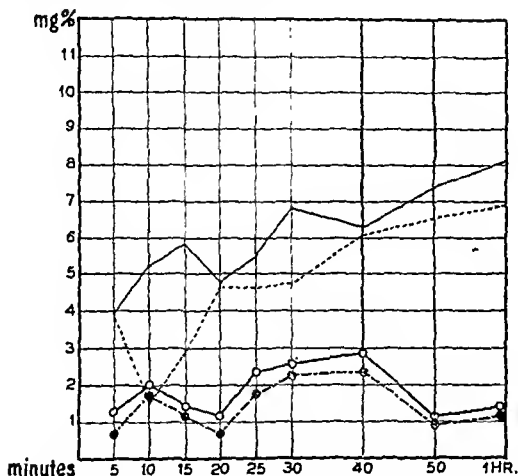


Chart 6.—Average free and total blood levels of sulfathiazole after a single dose of $\frac{1}{2}$ Gm. per kilogram of sulfathiazole administered by gavage.

--- Total Without pyloric occlusion
— Free

Among those animals with pyloric occlusion to which sulfathiazole was administered, four had free blood levels continuing until death below 2.0 mg. per 100 c.c. with but one exception, a temporary rise to 3.6 mg. per 100 c.c. at ten minutes. A fifth animal presented a continued rise in the blood level until death at forty minutes, at which time it had reached 7.0 mg. per 100 c.c. The three animals without pyloric occlusion had free blood levels ranging from 1.6 mg. per 100 c.c. at ten minutes, to 9.0 mg. per 100 c.c. at one hour. In two of these animals, the blood levels fluctuated, while the third had a steady rise to 9.0 mg. per 100 c.c. until death at sixty minutes.

Sodium Sulfathiazole (Charts 7 and 8).—In those animals to which sodium sulfathiazole had been given and in which pyloric occlusion had been done, blood levels of free sulfathiazole in each of four animals were respectively

3.2, 4.0, 4.7, and 7.3 mg. per 100 c.c. at five minutes after administration; whereas, in three without pyloric occlusion, the free levels were respectively 0.5, 3.2, and 8.8 mg. per 100 c.c.

In two of three animals with pyloric occlusion in which conjugated levels were determined at five minutes after administration of sodium sulfathiazole, there was conjugated as well as free sulfathiazole. In each of three determinations in those without pyloric occlusion both the free and conjugated forms were present.

Determinations of the blood sulfathiazole level in one animal with pyloric occlusion, at one minute, two minutes, and five minutes after administration of sodium sulfathiazole, were respectively 3.24, 3.50, and 3.10 mg. per 100 c.c. The

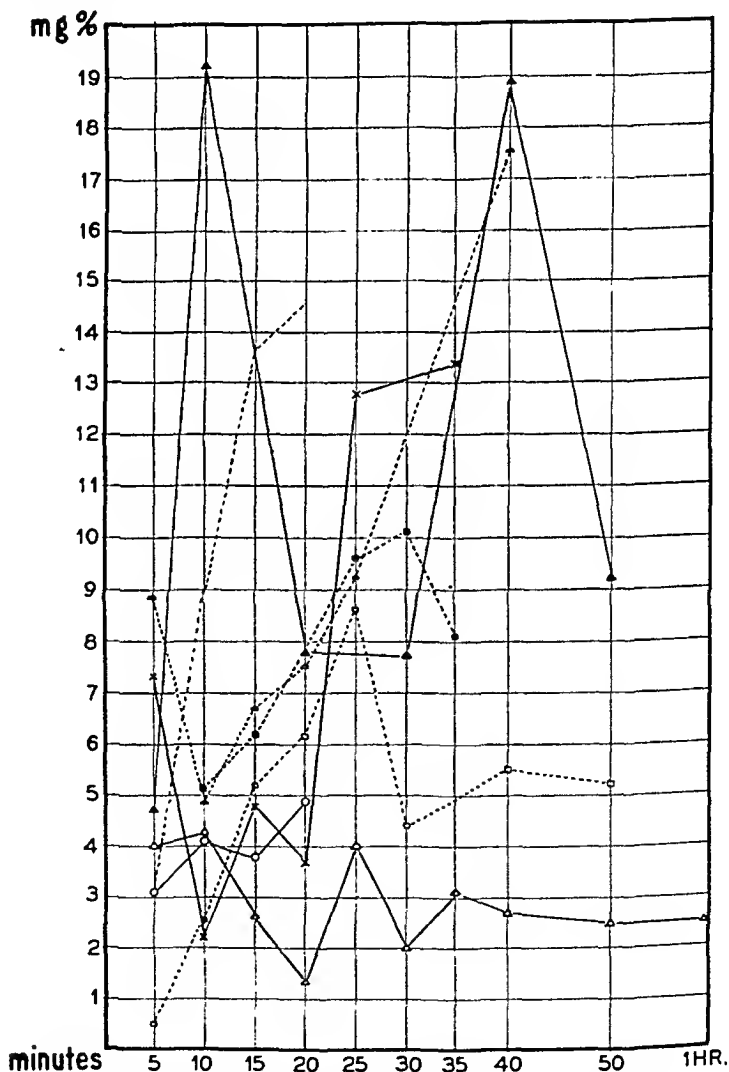


Chart 7.—Free blood levels of sodium sulfathiazole after a single dose of $\frac{1}{2}$ Gm. per kilogram of sodium sulfathiazole administered by gavage.

— Without pyloric occlusion.
 — With pyloric occlusion.

total drug at two minutes was 4.98 mg. per 100 c.c. Determinations of the total drug could not be made at one and five minutes.

In the animals to which sodium sulfathiazole had been administered, it was found that in those with pyloric occlusion, the levels of free drug in the blood, although they fluctuated widely, were similar to those obtained in those animals without pyloric occlusion. The amount of conjugated drug in those with

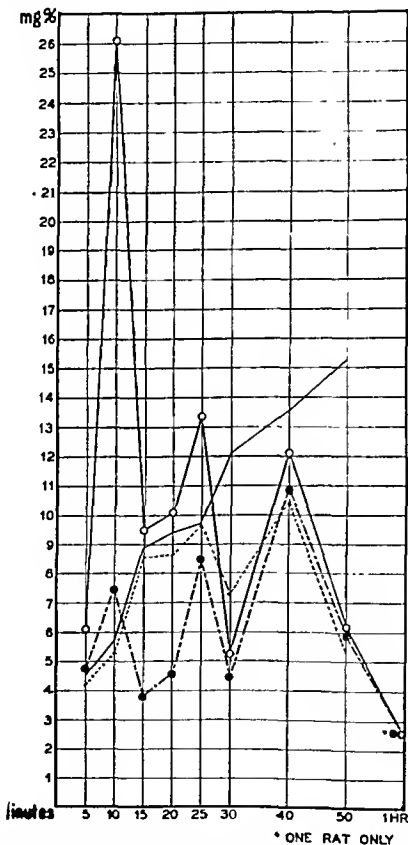


Chart 8—Average free and total blood levels of sodium sulfathiazole after a single dose of $\frac{1}{2}$ Gm. per kilogram of sodium sulfathiazole administered by gavage.

--- Total Without pyloric occlusion
 --- Free
 o--o Total With pyloric occlusion
 •---• Free

occlusion of the pylorus, however, while high at first, diminished rapidly, so that only a slight difference existed at thirty minutes and thereafter. In those without pyloric occlusion, the amount of conjugated drug increased progressively.

Sulfadiazine (Charts 9 and 10).—Sulfadiazine was present in the blood (0.9, 1.3, 1.5, and 1.5 mg. per 100 c.c.) in all four rats with pyloric occlusion five minutes after it had been given, and in both rats without pyloric occlusion (0.5 and 1.5 mg. per 100 c.c.).*

Three of the four rats without pyloric occlusion had conjugated as well as free sulfadiazine in the blood, and one of two without pyloric occlusion had conjugated drug at five minutes.

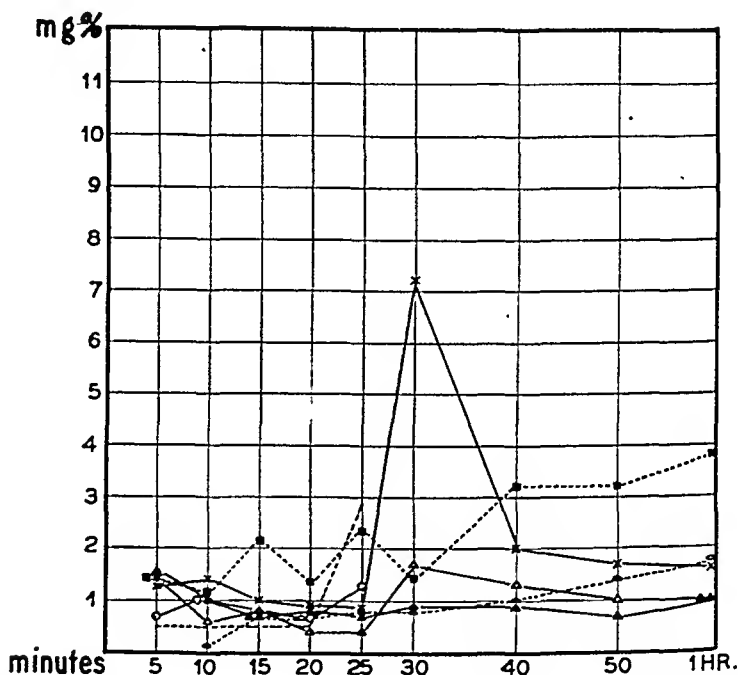


Chart 9.—Free blood levels of sulfadiazine after a single dose of $\frac{1}{2}$ Gm. per kilogram of sulfadiazine administered by gavage.

--- Without pyloric occlusion.
 — With pyloric occlusion.

In one rat with pyloric occlusion determinations for sulfonamide in the blood were made at intervals of one minute during the first five minutes. These revealed free blood levels of 0.9, 0.6, 0.9, 0.8, and 1.5 mg. per 100 c.c., respectively, at one, two, three, four, and five minutes. Levels of conjugated drug found at the first, second, third, fourth, and fifth minute periods were 1.54, 1.46, 1.92, 1.73, and 2.32 mg. per 100 c.c.

In subsequent determinations made during the remainder of the hour, it was found that the levels in the four animals with pyloric occlusion were closely similar to those without pyloric occlusion during the first twenty-five minutes. After this time, in three of the animals with pyloric occlusion, two

*No blood could be obtained at five minutes from the third animal without pyloric occlusion.

of which survived for one hour, the levels were similar to those without occlusion. The third rat with pyloric occlusion showed a rapid rise in free level of sulfadiazine in the blood from 0.9 mg. per 100 c.c. at twenty-five minutes to 7.2 mg. at thirty minutes, and a subsequent fall to 2.0 mg. at forty minutes; thereafter, the levels in the blood coincided with the trend of the rest of the group. One of the two animals without pyloric occlusion had a gradual rise in free blood sulfadiazine from 1.5 mg. per 100 c.c. at thirty minutes to 3.4 mg. per 100 c.c. at one hour. The other attained free blood sulfadiazine levels similar to those found in the animals with pyloric occlusion.

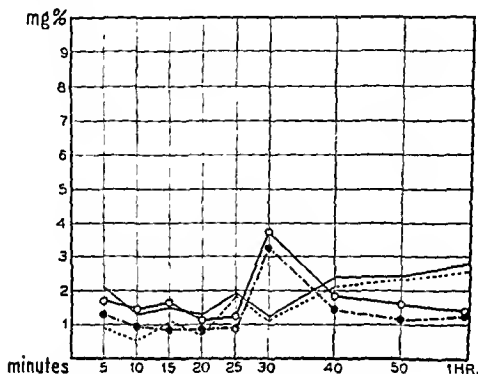


Chart 10.—Average free and total blood levels of sulfadiazine after a single dose of $\frac{3}{4}$ Gm. per kilogram of sulfadiazine administered by gavage.

--- Free Without pyloric occlusion
 --- Total Without pyloric occlusion
 o-o Free With pyloric occlusion.
 •-•-• Total With pyloric occlusion.

Sodium Sulfadiazine (Charts 11 and 12).—Following the administration of sodium sulfadiazine, all animals with pyloric occlusion had free sulfadiazine in the blood (0.3, 1.1, 1.3, 2.0, and 3.1 mg. per 100 c.c.) at five minutes. Very much greater levels were obtained in two animals without pyloric occlusion (10.6 and 13.1 mg. per 100 c.c.).*

Three animals with pyloric occlusion survived for fifty minutes or longer. Two had levels of 1.0 mg. per 100 c.c. or below. A third had levels between 2.5 and 6.0 mg. per 100 c.c. Two of the animals without pyloric occlusion had very high free levels. One had a progressive rise, which at first was sudden, from 13.0 mg. at five minutes to 27.2 mg. per 100 c.c. at fifty minutes. Another free sodium sulfadiazine level, after reaching 23.0 mg. per 100 c.c. at twenty minutes fell quickly to 7.8 mg. per 100 c.c. and then again rapidly rose to 15.4 mg. per 100 c.c. at the end of one hour. The free blood sulfadiazine level in a third animal initially was 10.6 mg. per 100 c.c. at five minutes, fell to 2.2 at twenty minutes, and 1.3 mg. at forty minutes; there then followed a gradual

*No blood was procured from a third animal at five minutes.

rise to 5.1 mg. per 100 c.c. at one hour. The degree of conjugation was slight in those animals with pyloric occlusion throughout the hour period. In those without pyloric occlusion, there was no conjugation during the first twenty minutes; however, during the next twenty minutes the average degree of conjugation increased chiefly because of lowered free levels, but from forty minutes to one hour there was less conjugation than during the previous period, with again slowly rising free levels which almost paralleled the total blood levels.

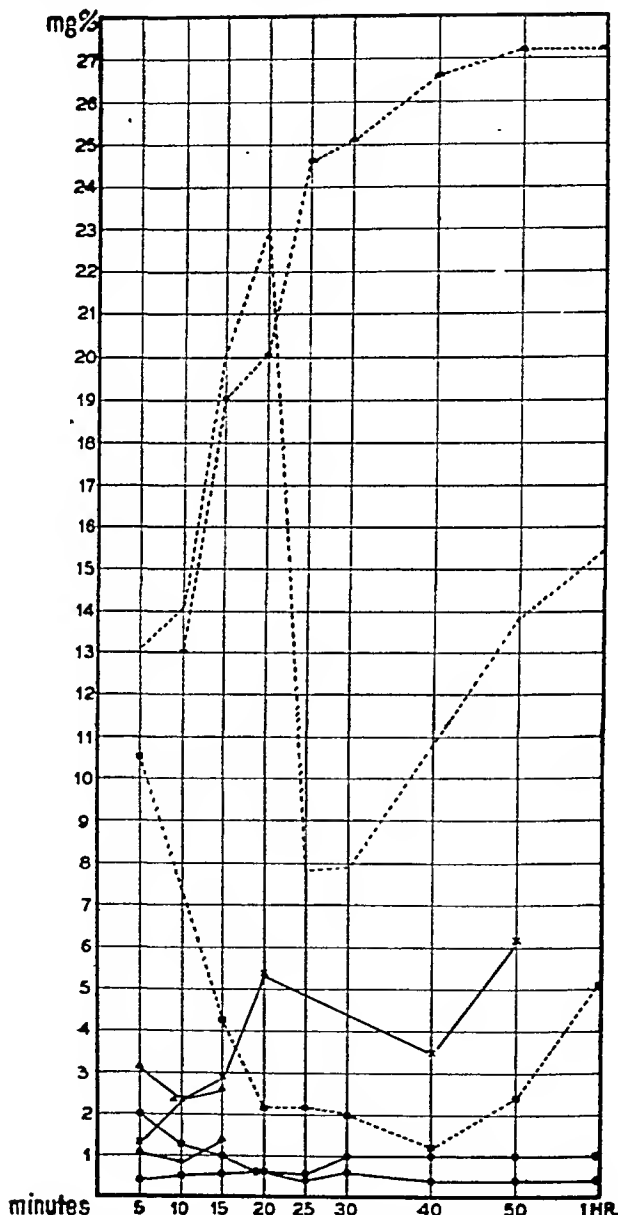


Chart 11.—Free blood levels of sodium sulfadiazine after a single dose of $\frac{1}{2}$ Gm. per kilogram of sodium sulfadiazine administered by gavage.

--- Without pyloric occlusion.

— With pyloric occlusion.

In another rat with pyloric occlusion to which sodium sulfadiazine had been given, free blood levels of 1.3, 1.4, and 1.2 mg. per 100 c.c. were obtained at one, four, and five minutes. Conjugated sulfadiazine, as well as free, was found at one and four minutes.

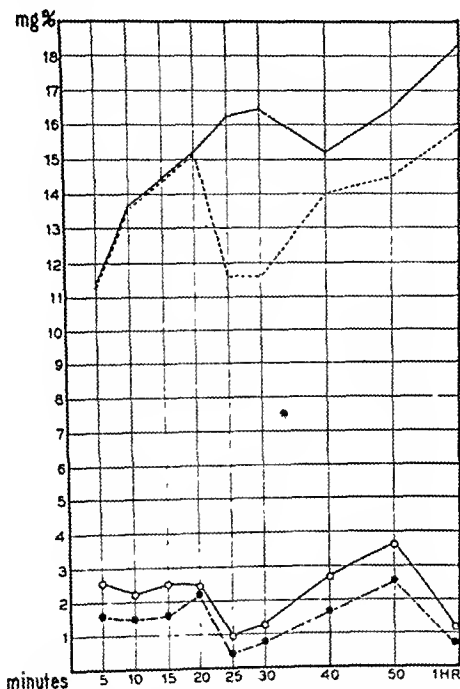


Chart 12.—Average free and total blood levels of sodium sulfadiazine after a single dose of $\frac{1}{2}$ Gm. per kilogram of sodium sulfadiazine administered by gavage.

— Total Without pyloric occlusion
 — Free Without pyloric occlusion
 o—o Total With pyloric occlusion
 ••••• Free With pyloric occlusion

SUMMARY

Study was made of the absorption of sulfapyridine, sodium sulfapyridine, sulfathiazole, sodium sulfathiazole, sulfadiazine, and sodium sulfadiazine as evidenced by blood level determinations made at short intervals during a one-hour period following their administration by gavage to two groups of albino rats. In one group, the animals were normal; in the other, mechanical occlusion of the pylorus had just been effected.

Sulfapyridine, sodium sulfapyridine, sulfathiazole, sodium sulfathiazole, sulfadiazine, and sodium sulfadiazine were absorbed rapidly in both groups of

animals as shown by their appearance in the blood in all determinations made at five minutes after their administration by stomach tube.

Six animals in the group with pyloric obstruction, each of which had received a different drug, and in which blood samples were taken at minute intervals for five minutes after administration, had significant quantities of the sulfonamide in the blood as early as one minute.

Conjugated forms of the sulfonamides were present as early as one minute after administration in four of these six animals; in the other two, they appeared at two minutes.

The majority of the animals in both groups had conjugated sulfonamide in the blood five minutes after administration.

Greater amounts of sulfapyridine and sulfathiazole were found in the blood of those animals without pyloric occlusion than in those rats with pyloric occlusion. There was no essential difference between the free and total blood levels of sulfadiazine in those with or without pyloric occlusion.

The average degree of conjugation was greater in most instances in those animals to which sodium sulfapyridine, sodium sulfathiazole, and sodium sulfadiazine were administered, than in those receiving respectively, sulfapyridine, sulfathiazole, and sulfadiazine.

The free and total blood levels closely paralleled each other in those animals, which received sulfapyridine and sulfadiazine, with and without pyloric occlusion. In those animals with pyloric occlusion which received sulfathiazole, the free and total levels, likewise, closely paralleled each other, but in those without occlusion moderately wide divergences occurred at irregular intervals.

The rats given sodium sulfapyridine and sodium sulfathiazole responded similarly whether or not pyloric occlusion had been effected. Except for occasional fluctuations, there were no significant differences in the free blood levels of these two groups. In the rats with pyloric occlusion, the total levels paralleled the free levels with temporary exceptions. In the animals without occlusion which received sodium sulfapyridine, the free and total levels closely paralleled each other during the first twenty-five minutes; the same was true for sodium sulfathiazole during the first fifteen minutes. After this, the free and total levels with both sodium sulfapyridine and sodium sulfathiazole became widely divergent with continued increase of the conjugated form.

The animals without occlusion to which sodium sulfadiazine was given had very much higher blood levels than those rats with pyloric occlusion, the differences between free levels ranging up to 15 mg. per cent. The levels of the free and total sulfadiazine in the blood paralleled each other in the rats with pyloric occlusion, but remained approximately the same in those without occlusion for the first twenty minutes; after that, a wide divergence occurred.

CONCLUSIONS

1. Sulfapyridine, sodium sulfapyridine, sulfathiazole, sodium sulfathiazole, sulfadiazine, and sodium sulfadiazine seem to be absorbed from the stomachs of albino rats in significant amounts. With the exception of sulfadiazine, however, significantly greater quantities of the sulfonamides are absorbed from the gastrointestinal tracts of those animals without pyloric occlusion.

2. Conjugation of the sulfonamides in albino rats occurs as early as one minute after administration by gavage.

REFERENCES

1. Peterson, O. L., Strauss, E., Taylor, F. H. L., Finland, M.: Absorption, Excretion and Distribution of Sulfadiazine. *Am. J. M. Sc.* 201: 357-367, 1941.
- Reinhold, J. G., Flippin, H. F., and Schwartz, L.: Observations on the Pharmacology and Toxicity of Sulfathiazole in Man. *Am. J. M. Sc.* 199: 393-401, 1940.
- Reinhold, J. G., Flippin, H. F., Schwartz, L., and Dunn, A. H.: The Absorption, Distribution, and Excretion of 2-Sulfanilamido Pyrimidine (Sulfapyrimidine, Sulfadiazine) in Man. *Am. J. M. Sc.* 201: 106-115, 1941.
- Barlow, O. W., and Climenko, D. R.: Studies on the Pharmacology of Sulfapyridine and Sulfathiazole. *J. A. M. A.* 116: 282-286, 1941.
- Marshall, E. K., Jr., Cutting, W. C., and Cover, W. L.: Absorption and Excretion of Certain Sulfanilamide Derivatives. *Bull. Johns Hopkins Hosp.* 63: 318-327, 1938.
- Marshall, E. K., Jr., and Cutting, W. C.: The Absorption and Excretion of Sulfanilamide in the Mouse and Rat. *Bull. Johns Hopkins Hosp.* 63: 328-336, 1938.
2. Sadusk, J. F., Jr., Blake, F. G., and Seymour, A.: Observations on the Absorption, Excretion, Diffusion and Acetylation of Sulfathiazole in Man. *Yale J. Biol. & Med.* 12: 681-696, 1940.
- Sadusk, J. F., Jr., and Tiedway, J. B.: Observations on the Absorption, Excretion, Diffusion, and Acetylation of Sulfadiazine in Man. *Yale J. Biol. & Med.* 13: 540-556, 1941.
- Ratish, H. D., Davidson, A., and Bullock, J. G. M.: The Absorption and Excretion of Sulfapyridine and of Sodium Sulfapyridine in Man. *J. Pharmacol. Exper. Ther.* 69: 365-374, 1940.
3. Loughlin, E. H., Bennett, R. H., Flanagan, M. E., and Spitz, S. H.: Relative Absorption and Conjugation of Nine Compounds by Humans During a Three-Hour Period. *Am. J. M. Sc.* 205: 223-229, 1943.
4. Marshall, E. K., Jr., Cutting, W. C., and Cover, W. L.: Absorption and Excretion of Certain Sulfanilamide Derivatives. *Bull. Johns Hopkins Hosp.* 63: 318-327, 1938.
5. Long, P. H., and Bliss, E. A.: The Clinical and Experimental Use of Sulfanilamide, Sulfapyridine, and Allied Compounds. New York, 1939, Macmillan.
6. Loughlin, E. H., Bennett, R. H., Flanagan, M. E., and Spitz, S. H.: Treatment of Experimentally Induced Type I Pneumonia in Albino Rats. *J. Lab. & Clin. Med.* 28: 1455-1461, 1943.
7. Bratton, A. C., and Marshall, E. K., Jr.: A New Coupling Component for Sulfanilamide Determination. *J. Biol. Chem.* 128: 537-550, 1939.

COLD AGGLUTININS IN TUBERCULOSIS

EZRA BRIDGE, M.D., ARELYN THURSTON, M.S., AND ANTHONY REPICCI,
ROCHESTER, N. Y.

RECENT investigations of cold agglutinins in the blood serum have shown that their presence may be used as a diagnostic test for atypical pneumonias.^{1, 2} This we have found to be true in a small number of cases which have been under supervision at this hospital. Still more recently, Siefert and Krautman³ reported cold agglutinins present in the serum of patients with tuberculosis. This possibility was thought worthy of investigation because of difficulties which might arise if both tuberculosis and atypical pneumonia should exist at the same time in the same individual. The report of our investigation is as follows:

SELECTION OF CASES

Fifty individuals were used for the tests. The selection was made on the basis of activity and extent of disease. The group thus selected was classified as follows: active, minimal, 15; active, moderately advanced, 16; active, far advanced, 13; inactive, moderately advanced, 1; nontuberculous, 5.

DONORS

Cells from four Group 0 donors were used. Three of these donors were nontuberculous, and one was classified as active, minimal tuberculosis. The serum of each patient tested was used with the cells of more than one donor, and donors' cells were tested with their own sera as well as the sera of patients.

TECHNIQUE

Each patient's serum was diluted with physiological saline in twelve tubes from a 1:2 to a 1:4,096 dilution. Five-tenths cubic centimeter of a 2 per cent suspension of washed human Group 0 cells was then added, mixed with the diluted serum and placed in the refrigerator at 0° C. temperature overnight. Readings were taken immediately upon removal from the refrigerator and one hour later (in positive cases).

RESULTS

In no instance were we able to demonstrate cold agglutinins in any of the sera of patients with tuberculosis only. In two instances, however, where atypical pneumonia as well as tuberculosis was present, positive reactions were recorded.

SUMMARY

In a series of fifty cases of tuberculosis the test for cold agglutinins was negative. This leads us to the opinion that tuberculosis in itself does not produce these agglutinins. We therefore conclude that the test has a distinct value in differentiating between tuberculosis and atypical pneumonia.

REFERENCES

1. Peterson, O. L.: Ham, T. H., and Finland, Maxwell: Cold Agglutinins (Autohemagglutinins) in Primary Atypical Pneumonias, *Science* 97: 167, 1943.
2. Horstman, Dorothy M., and Tatlock, Hugh: Cold Agglutinins as a Diagnostic Aid, *J. A. M. A.* 122: 369, 1943.
3. Siefert, R. S., and Krautman, B.: Cold Hemagglutinin Reactions in Tuberculosis, *J. LAB. & CLIN. MED.* 29: 270, 1944.

THE INCIDENCE OF PLASMA-COAGULATING STAPHYLOCOCCI IN THE FECES OF CHRONIC INVALIDS

GEORGE H. CHAPMAN, NEW YORK, N. Y.

IT IS possible¹ to detect plasma-coagulating staphylococci in feces by plating the sample on bromthymol blue lactose agar to which has been added potassium tellurite (either 0.10 c.c. of 0.10 per cent tellurite added to the surface of a previously poured plate or, better still, 0.10 c.c. of 5.0 per cent unheated tellurite added to 250 c.c. of bromthymol blue lactose agar just before pouring).

In studying the feces of 124 chronic invalids, plasma-coagulating staphylococci were found in 12 (9.7 per cent) in the following amounts: "moderate number," "moderate number," 1, 1, 2, 2, 2, 7, 7, 10, 10 and 14 millions per 100 grams of dry feces. There was an overgrowth of other organisms in 8 specimens (6.5 per cent).

There was a relationship to the presence of staphylococci in the upper respiratory tract, plasma-coagulating staphylococci being found in the pharynx in 11, and in both pharynx and nares in 9 of the 12 that showed them in the feces. This indicates that the organisms had been swallowed. Not all swallowed staphylococci, however, reach the feces because, of the 44 patients that showed plasma-coagulating staphylococci in the pharynx, only 11 showed them in the feces.

The method does not entirely eliminate other organisms but the number of other organisms is so reduced and the appearance of colonies of plasma-coagulating staphylococci is so characteristic that there is little difficulty in recognizing them. Enterococci are the most common interfering organisms but the colonies are quite different from those of staphylococci. Five of the cultures of staphylococci isolated failed to clot plasma and are not included in the above calculations.

REFERENCE

1. Chapman, G. H.: The Isolation of Pathogenic Staphylococci From Feces, *J. Bact.* 47: 211, 1944.

ALLERGY FROM TIMBÓ (LONCHOCARPUS H.B.K.)

REPORT OF A CASE

A. OLIVEIRA LIMA, M.D., RIO DE JANEIRO, BRAZIL

CONSIDERING the wide use of "timbó" (*Lonchocarpus* H.B.K.) and "eubé" (*Derris* Lour.) as insecticides, the paucity of reports of allergic manifestations in connection with these substances is rather surprising. Weston¹ has reported two cases of asthma due to *Derris*-root in flea powder.

We had the opportunity of studying a patient with allergy due to timbó who manifested both asthma and dermatitis of the contact type. The interest of this case lies in the demonstration of cross reactions between the atopens of *Lonchocarpus* (timbó) and *Derris* (eubé), plants of the Leguminosae family.

CASE REPORT

M. J. S., a Brazilian white male, aged 33 years, has been working as an engineer for three years, on the road-building service in the State of Amazonas (North Brazil). Until he started to work there, he had never suffered from asthma or eczema. He had had urticaria when he ate shrimps. Typhoid fever and pneumonia were the only illnesses he had had in the past. His mother had suffered from allergic rhinitis; two maternal aunts had asthma; one sister and two sons had chronic urticaria.

In the beginning of 1940 his work brought him into close contact with the flora of Amazon. His abode was located at a distance of some kilometers from where he worked and he was compelled daily to walk through dense shrubs and forests. The great number of mosquitoes of this region forced him to spray his house with a mixture of kerosene and timbó powder. He noticed first that he could not breathe well when he stayed in the rooms which had been sprayed. Without paying special attention to the fact he continued to use timbó, the only insecticide at his disposal.

In May, 1940, there appeared the first trouble which forced him to stop using the spray. At that time, inhalation of the above mixture would cause him to sneeze, followed by coryza, pruritus of the nose and conjunctiva, oppression on the chest, shortness of breath and wheezing. On stopping use of the insect powder, the patient was relieved of all symptoms. Although he no longer sprayed his rooms, he could not altogether avoid the effects of the mixture, for his colleagues, at whose houses he called, kept on spraying. Frequently he felt himself suddenly compelled to leave the houses of friends because of violent attacks of asthma.

At that time he experienced pruritus and reddening of the skin of his hands while working in the field. After a few days, there appeared small vesicles, followed by pruritus and eczematization of his hands and forearms. Although he suspected the toxic action of some plant, he was unable to identify it. His eczema healed after ten days' rest. On returning to work the eruptions reappeared, of the same character, but now spreading beyond the elbows. The patient came to Rio de Janeiro where we examined him. His dermatosis had healed during the journey.

From the Laboratorio de Alergia São Borja, Rio de Janeiro, Brazil.
Read before the Centro de Estudos de Alergia do Rio de Janeiro, Jan. 23, 1944.
Received for publication, Feb. 24, 1944.

Physical Examination.—The examination did not reveal anything of interest. Kuhn test was negative. He had a blood eosinophilia of 7 per cent. Urine and stool examinations were negative.

Skin Tests.—Scratch and intradermal tests were negative for foods, common inhalants, fungi and pollens. The scratch tests with a 1 per cent extract of *Lonchocarpus nicou* (timbó) root-powders and with a similar extract from *Derris elliptica* (eubé) were both positive 3 plus. The same tests were negative on the skin of three normal persons. The passive transfer tests were positive 2 plus on the skins of 2 non-atopic recipients. Scratch and intradermal tests with an extract made from *Tephrosia*, a genus of the Leguminous family and possessing also the insect-killing capacity, were negative. Also negative were the scratch and intradermal tests done with a standardized extract from pure rotenone. Patch tests, not covered, with crude extracts obtained from the leaves of *Lonchocarpus* and *Derris*, gave positive reactions of the same intensity for the two genera. After 24 hours there were intense erythema, distinct infiltration and vesiculation, within an area 3 centimeters in diameter. On the third day after performance of the patch test, an erythematous "flare-up" appeared on the backs of the two hands, which disappeared without vesiculation after 72 hours. The same tests were negative on the skins of 3 normal persons. Attempts to transfer the sensitivity in relation to the resinous principles of the two plants have not been successful. The patch test performed with *Tephrosia*, pure rotenone, *Lithrea molleoides*, and kerosene were negative. The contact tests with powdered leaves or roots of *Lonchocarpus* and *Derris* exhaustively treated with ether (tests performed with a 50 per cent ointment by weight of powder and petrolatum) were wholly negative after 24, 48, 72 hours and after 7 days.

DALE EXPERIMENTS

In order to be certain of the immunological relationship between the water soluble antigens of the two genera, *Lonchocarpus* and *Derris*, a series of experiments with the Dale test were conducted. Five guinea pigs were sensitized by means of a single intraperitoneal injection of a purified alum-precipitated extract. It was possible to inactivate completely a *Lonchocarpus nicou* sensitized uterine strip by means of an extract of *Derris elliptica* and vice-versa, in several cross neutralization experiments. Similar experiments were carried out with the species* *Lonchocarpus urucú* Kill, *L. maculatus* DC., *L. densiflorus* Benth., *L. floribundus* Benth., *L. sericeus* H.B.K., *L. denudatus* Benth., *L. spruceanus* Benth., *L. negrensis* Benth.; *Derris guianensis* Benth., *D. negrensis* Benth., *D. longifolia* Benth. All of them showed the presence of a common antigen.

Extracts from three species of the genus *Tephrosia* Pers. (*T. toxicaria* Pers., *T. brevipes* Benth., *T. nitens* Benth.) and from pure rotenone, failed to produce contraction on the uteri sensitized to *Lonchocarpus* or *Derris*.

Precipitin tests were performed with the extracts used for sensitization in sera from the five guinea pigs, but the results were negative.

DISCUSSION

The simultaneous appearance, in our patient, of asthma and dermatitis of the contact type, must be considered as a mere coincidence. The eczematous eruptions have been found only in connection with the resinous substance of the *Lonchocarpus* and *Derris* leaves. The patch tests with leaf and root powder, exhaustively extracted with ether, did not produce any reaction. We must conclude that the eczematous eruptions cannot be ascribed to the atopen prin-

*All the species of the genus *Lonchocarpus* are designated here as "timbó" and are generally considered as containing rotenone.

ciple of the plants just considered. Besides, the aspect of the lesions is quite different from that described by Walzer and Albert.²

The existence of cross reactions between the atopenic principles of the two genera, *Lonchocarpus* and *Derris*, has been demonstrated by means of the Dale test. Other genera of the Leguminosae family have been shown incapable of neutralizing a *Lonchocarpus* or *Derris* sensitized uterine strip. The fact that our patient does not react to pure rotenone, one of the principal substances endowed with toxic action for cold-blooded animals, shows that the atopenic and contact principles of the two genera belong to different classes of substances. The other substances which are contained in timbó powder (deguelina, tephrosina, toxiearol) could not be investigated owing to the difficulty of obtaining them in the pure state. We have not been able to study the immunologic relationships of the resinous substances of the genera *Lonchocarpus* and *Derris*. The attempts artificially to sensitize the skin of three normal persons with its resin did not give conclusive results.

SUMMARY

The writer cites the case of a patient who became allergic, at the same time, to the atopenic and resinous principles of timbó, a plant belonging to the genus *Lonchocarpus*. The patient reacted specifically also to the two principles of the genus *Derris* (enbê). On studying the antigenic behavior of the atopenic principle of the genera *Lonchocarpus* and *Derris* by means of the Dale test, the writer was able to show the existence of cross reaction between the two plants. Pure rotenone was inactive in the cutaneous tests and indifferent to the uterine muscle of guinea pig sensitized to any of the above genera referred to.

We wish to express our thanks to Professor Mello Barreto and to Dr. Luiz de Mello Filho for the facilities they afforded us in the execution of this work.

REFERENCES

1. Weston, C. G.: *Derris-root, a New Allergen*, *J. Allergy* 8: 62, 1937.
2. Albert, M., and Walzer, M.: *Contact-Reactions in Atopy. II. The Incidence of Contact Reactions With Various Allergens*, *J. Invest. Dermat.* 3: 119, 1940.

always present on the slide. The fragility of red cells to hypotonic saline was tested in the conventional manner.¹⁰ Total hemoglobin, methemoglobin, and sulfhemoglobin were determined by the method of Evelyn and Malloy.¹¹ Plasma bilirubin was measured by the method of Malloy and Evelyn.¹² At the end of the experiments most of the animals were sacrificed, usually by decapitation, and tissues were obtained immediately for bone marrow examination.

The significance of the observed hematologic changes in peripheral blood was established largely by comparison of the control observations on each animal or group of animals with the subsequent experimental values. In parallel experiments in which four animals or more were employed in a group, all control determinations were pooled, and the mean with its standard error was calculated. The experimental values of the group at any given time interval during administration of the drugs were similarly treated. Statistical significance of the difference between the means was tested by Fisher's "t" test.¹³ (This treatment was applied especially to the "Series 2" groups, Figs. 1 and 3.) The normal values reported for the eighty-one animals examined in this laboratory⁹ and a smaller group of ten animals, followed for several months were also employed in a general way in interpreting the data. The myeloid-erythroid ratios calculated from the bone marrow counts (Table II) were also treated statistically as described above except that the control values in this case were derived from a separate group of animals.⁹

RESULTS

I. ACETANILID.—

Series 1.—Two dogs received acetanilid 9 mg. per kilogram daily for 147 days; the drug was discontinued for two weeks, and the animals sacrificed. Two other dogs received progressively increasing doses of acetanilid: 9 mg. per kilogram daily for 96 days, 18 mg. per kilogram for 51 days, 36 mg. per kilogram for 63 days, 72 mg. per kilogram for 77 days, and 144 mg. per kilogram for 45 days; i.e., 332 days of continuous medication. The hematologic data are summarized in Table I. The chronic administration of 9, 18, and 36 mg. per kilogram of acetanilid produced no significant changes in the peripheral blood. An increase in dosage to 72 mg. per kilogram resulted in a slight decrease in total hemoglobin, erythrocytes, and cell volume in Dog 15 but no change in Dog 19. A further increase in dosage to 144 mg. per kilogram, however, produced in both dogs a moderate decrease in total hemoglobin, erythrocytes, and cell volume, and a significant reticulocytosis. In this entire series no significant changes occurred in the total or differential leucocyte counts. There was no change in fragility to hypotonic saline and no cumulation of methemoglobin or sulfhemoglobin. The color and volume indices remained normal. The animals maintained or gained weight.

Series 2.—Acetanilid 36 and 72 mg. per kilogram. Two groups of four animals each were on experiment for 78 days; one group received 36 mg. per kilogram per day and the other group 72 mg. per kilogram per day. Blood examinations were made each week. The data on peripheral blood are summarized in Fig. 1. No consistent change occurred in total hemoglobin, erythrocytes, or cell volume in the 36 mg. per kilogram group, although a few of the values for erythrocytes were significantly reduced. The color and volume indices

remained normal. The values for total hemoglobin, erythrocytes and cell volume for the 72 mg. per kilogram group showed phasic decreases which in general were quite significant. At the end of the experiment the mean values were significantly reduced. The color index remained normal but the volume index increased slightly. In both groups there was a small increase in the reticulocyte

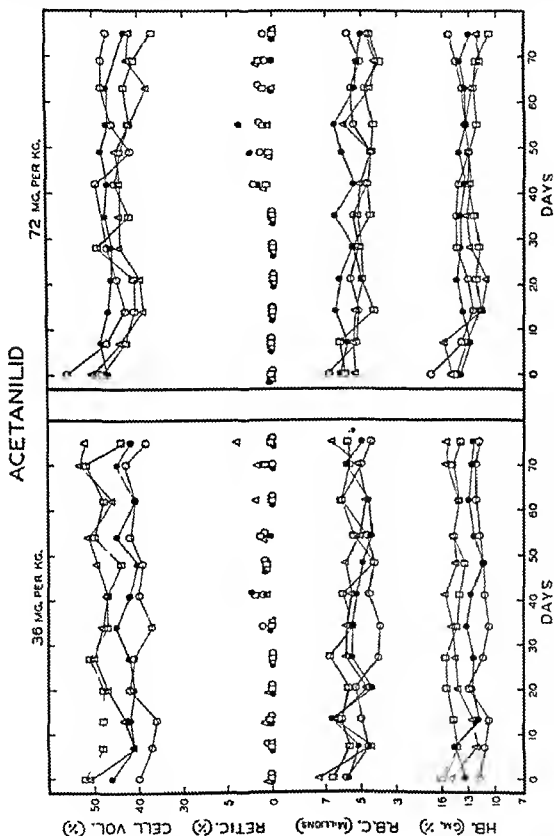


Fig. 1.—Effect of daily administration of acetanilid 36 mg. per kilogram (left) and 72 mg. per kilogram (right) on peripheral blood. Each character represents an individual dog.

count beginning about the fortieth day. No significant deviation was observed in the total or differential leucocyte count, and no cumulation of methemoglobin or sulphemoglobin occurred. All animals either maintained or gained weight.

Series 3.—Acetanilid in large doses. Experiments were carried out on twelve dogs using, in most instances, a dose of 250 mg. per kilogram per day

for five to nine days. Occasionally the experiment was initiated by two 250 mg. per kilogram doses on the first day. The peripheral blood was usually examined daily and administration of the drug stopped after a severe anemia developed. Some animals were sacrificed at this time for bone marrow examination; others were followed to complete recovery of the peripheral blood picture. In several instances the latter type of experiment was repeated one or more times on the same animal.

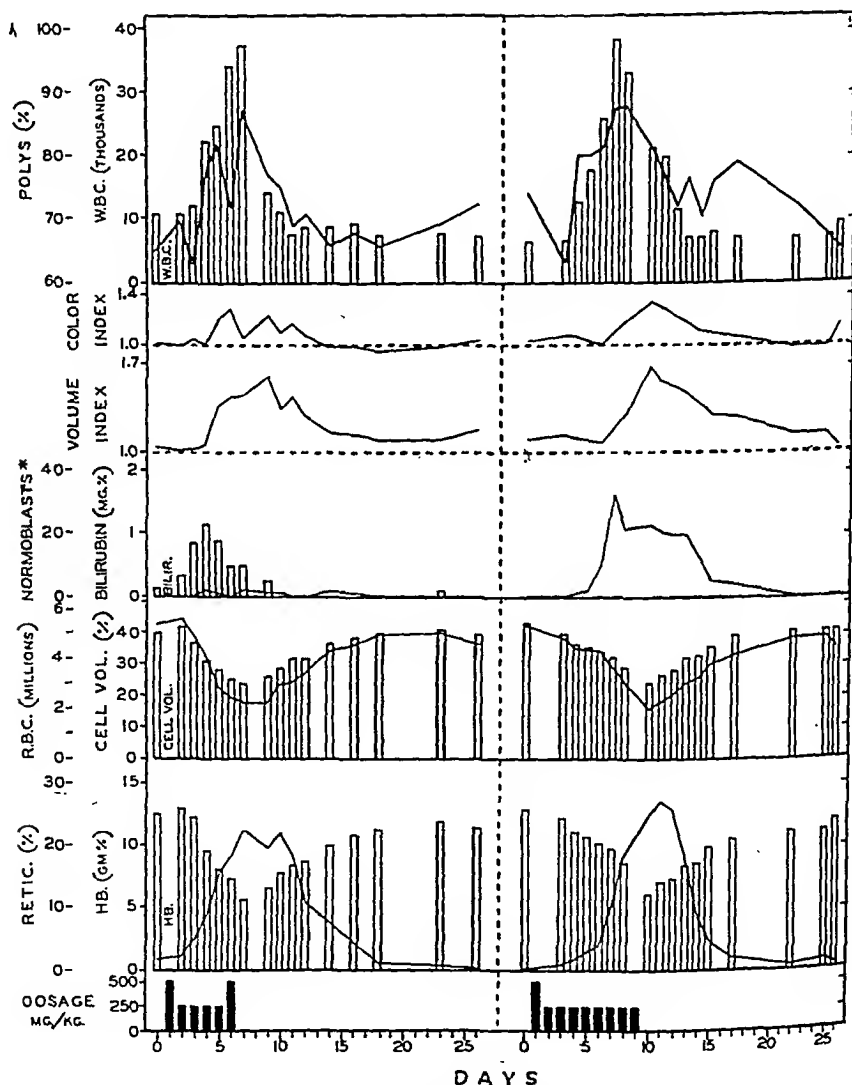


Fig. 2.—Effect of the administration of large doses of acetanilid on peripheral blood: the first experiment (left), and third experiment (right) on the same dog. The inner ordinate refers to the bars. The number of normoblasts are those observed in counting 200 leucocytes; the leucocyte counts are uncorrected and are thus too high by the number of normoblasts present.

Two examples of the effects produced with these large doses of acetanilid on one dog are shown in Fig. 2. In this animal the experiment was repeated four times, the first and third experiments being shown. In the first experi-

ment this dog received 500 mg. per kilogram on the first day, 250 mg. per kilogram on the second, third, fourth, and fifth days, and 500 mg. per kilogram on the sixth day. It is evident that by the fourth day a definite decrease in total hemoglobin, erythrocytes, and cell volume had occurred which reached a minimum on the seventh day. The reticulocyte response began on the third day. When administration of the drug was stopped recovery was rapid and normal values were approximated by the twenty-third day. The anemia was macrocytic and tended to be slightly hyperchromic. The development of the anemia was associated with a mild bilirubinemia, and some increase in the number of normoblasts in the peripheral blood.* During the last few days of administration of the drug and associated with the development of the anemia, there occurred a marked leucocytosis and an increase in the percentage of polymorphonuclear neutrophils with a definite shift toward more immature forms as indicated by a decrease in the polynuclear count. Younger forms of lymphocytes also appeared. The leucocytosis subsided early in the recovery period. The third experiment, Fig. 2, and the fourth experiment were in general a reproduction of the events of the first. The drug was administered for nine and five days, respectively. The chief variation from the first experiment was the appearance of a greater number of normoblasts in the blood. The second experiment on this animal differed only quantitatively in that there was a lesser tendency to the development of anemia.

The results shown in Fig. 2 may be taken as representative of this series. Some variation was noted in the number of daily 250 mg. per kilogram doses required to produce a severe anemia, although five to ten days were usually sufficient. Similar experiments were also made on four splenectomized dogs. The hematologic effects were essentially the same and no definite difference in susceptibility or rate of recovery was noted. No excretion of methemoglobin or sulfhemoglobin was observed in any of these experiments.

Effect of Acetanilid on Bone Marrow.—Examination of femur sections and imprints of the marrow from dogs in Series 1 (9 mg. per kilogram) revealed normal cellularity with a slightly increased percentage in the erythroid series for Dog 9 (confined chiefly to the polychromatic normoblasts), and no change in Dog 10. The femur marrow imprints of Dog 15 (progressive 9 to 144 mg. per kilogram) showed an increased erythroid percentage with a myeloid-erythroid ratio of 0.904. Femur sections on both Dogs 15 and 19 showed an increase in erythroid cells and some hyperplasia.

The result of differential rib marrow counts for Series 2 are shown in Table II. With the 36 mg. per kilogram dose the average myeloid-erythroid ratio for the rib was 0.946 and for the femur 0.827, representing a significant decrease from the control series. This was associated with an increase in the pronormoblasts and orthochromatic normoblasts. The femur marrow sections also indicated an increase in the erythroid series; hyperplasia was evident in two animals, no change in one, and an apparent hypoplasia in one. The effect of the 72 mg. per kilogram dose was even more significant. The average myeloid-erythroid ratio was reduced to 0.817 for the rib preparation and 0.647 for the femur. The increase in erythroid percentage occurred chiefly in the polychromatic and orthochromatic normoblasts. The histologic sections also

*We have frequently noted occasional normoblasts in the peripheral blood of control dogs.

TABLE II

EFFECT OF ACETANILID AND ACETOPHENETIDIN ON DIFFERENTIAL BONE MARROW COUNTS FROM RIB SMEARS IN THE DOG

TYPE OF CELL	CONTROLS* 8 DOGS	ACETANILID 36 MG./KG. 4 DOGS	ACETANILID 72 MG./KG. 4 DOGS	ACETO- PHENETIDIN 60 MG./KG. 4 DOGS	ACETO- PHENETIDIN 120 MG./KG. 5 DOGS
Myeloblasts	0.2-1.0 0.6	0.4-0.7 0.6	0.5-1.0 0.8	0.7-1.5 1.1	0.4-1.9† 1.0‡
Promyelocytes	0.7-2.8 1.6	0.3-1.4 1.0	0.5-0.9 0.7	1.0-1.5 1.2	0.7-2.3 1.5
Myelocytes	2.7-10.0 6.0	1.9-3.5 2.8	2.0-4.1 2.6	3.3-5.7 4.2	3.6-8.1 5.8
Metamyelocytes	1.1-4.6 3.4	1.1-4.3 2.7	2.0-3.6 3.0	2.8-5.2 3.4	1.6-4.7 3.6
Bands, Neutrophilic	6.8-17.4 11.7	9.3-10.2 9.8	6.7-9.8 8.0	7.6-12.9 9.8	6.4-11.2 8.3
Segmenters, Neutrophilic	16.8-44.2 30.1	22.3-29.6 26.8	21.4-29.3 25.9	19.8-27.9 22.6	21.4-33.1 24.5
Eosinophils	0.4-3.8 2.0	1.8-4.8 2.7	0.9-1.9 1.4	0.7-2.2 1.5	0.9-2.8 1.7
Monocytes	0.0-0.3 0.2	0.0-1.0 0.1	0.0-0.1 0.0	0.0-0.3 0.1	0.0-0.3 0.1
Lymphocytes	0.2-2.7 0.9	0.7-2.0 1.2	0.9-2.3 1.5	0.5-0.7 0.6	0.5-1.5 0.9
Pronormoblasts	0.2-1.2 0.6	1.3-1.4 1.4	0.9-1.7 1.4	1.0-1.4 1.2	0.7-1.6 1.1
Normoblasts, Basophilic	6.4-9.9 7.8	7.1-8.2 7.5	5.6-9.8 7.4	8.5-9.3 8.9	9.9-13.9 11.6
Normoblasts, Polychromatic	11.0-26.0 16.4	17.2-19.2 18.1	20.3-22.4 21.3	18.7-21.7 19.8	14.3-23.9 20.8
Normoblasts, Orthochromatic	8.9-25.8 17.4	18.9-27.8 23.9	17.3-28.5 24.5	18.1-27.6 24.2	10.8-24.8 17.7
Megakaryocytes	0.0-1.4 0.5	0.2-0.3 0.2	0.0-0.4 0.2	0.0-0.2 0.1	0.0-0.3 0.2
Unclassified	0.8-1.4	1.0-1.9	1.1-1.8	1.1-1.5	0.8-1.5
M-E Ratio Rib	0.6-1.78 1.37	0.83-1.16 0.946	0.68-1.08 0.817	0.75-1.09 0.836	0.77-1.17 0.935
M-E Ratio Femur Imprints	1.06-2.39 1.610	0.70-1.05 0.827	0.50-0.73 0.647	0.68-0.96 0.768	0.67-1.48 1.029

*See reference 9.

†Range.

‡Mean.

revealed an increase in the erythroid series. Hyperplasia of the marrow was evident in three animals with no change in the fourth.

A number of bone marrow observations were made on animals from Series 3, the animals which had received large doses of acetanilid sufficient to produce a peripheral blood picture similar to that demonstrated in Fig. 2. For example, a dog that had received 250 mg. per kilogram of drug per day for six days was sacrificed on the eleventh day with peripheral blood values as follows: total hemoglobin 7.5 Gm. per cent, erythrocytes 3.13 million, cell volume 30 per cent, reticulocytes 33.3 per cent, color index 1.01, and volume index 1.31. The bone marrow differential counts showed a marked decrease of the myeloid-erythroid ratio with values of 0.451 for rib and 0.394 for femur. An increase in all of the erythroid series was evident, but was especially marked in the polychromatic normoblasts (40.6 per cent). Both the white and red series of cells were complete. Histologic sections showed hyperplasia of the femur marrow.

II. ACETOPHENETIDIN.—

Series 1.—Acetophenetidin was administered to six dogs in 15 mg. per kilogram doses for 43 to 150 days. Five of these animals were then continued on 30 mg. per kilogram for 16 to 102 days; then four of these animals continued on 60 mg. per kilogram for 39 to 46 days; and finally two of the animals were continued on 120 mg. per kilogram for 28 days.

TABLE III
EFFECT OF DAILY ADMINISTRATION OF ACETOPHENETIDIN IN THE DOG

DOG NO.	DAYS	DAILY DOSAGE MG./KG.	Hb GM %	ERYTHROCYTES IN MILLIONS	CELL VOLUME %	RETICULO-CYTES %	LEUCOCYTES IN THOUSANDS
27	0	0	13.6	6.58	49.5	0	18.30
	76	15	18.7	7.11	44.0	0	8.25
26	0	0	13.7	6.01	41.5	0.2	9.74
	76	15	11.9	5.19	36.0	0	11.40
	71	30	11.5	5.01	35.0	0.4	20.83
28	0	0	15.4	7.23	47.5	0	12.60
	133	15	13.9	6.72	56.9	0.6	13.05
	51	30	14.0	6.47	50.5	1.1	11.70
	45	60	14.2	7.13	49.0	0.1	10.33
29	0	0	14.4	6.28	46.0	0.01	17.17
	96	15	12.5	4.95	37.0	1.0	14.15
	97	30	12.1	5.02	40.0	0.7	17.83
	45	60	10.5	4.70	35.0		12.78
P-1	0	0	15.5	6.38	46.0	0.3	10.30
	41	15	16.2	7.75	48.0	0.6	12.53
	14	30	15.5		47.0	0.4	8.23
	33	60	15.6	5.95	44.0	2.1	9.25
	27	120	14.2	5.14	42.0	0	7.40
P-2	0	0	15.8	5.95	41.0	0.6	10.19
	41	15	16.0	8.12	50.0	0.9	10.70
	14	30	13.9		44.0	0.2	12.60
	35	60	17.3	6.29	51.0	0.6	9.18
	27	120	17.1 ?	5.68	45.0	0.8	8.78

The data as summarized in Table III did not demonstrate any consistently significant change in total hemoglobin, erythrocytes, and cell volume, although certain of the individual values were below the control level. In this entire series no significant changes occurred in the total or differential leucocyte count. There was no change in fragility to hypotonic saline or cumulation of methemoglobin or sulfhemoglobin. The color and volume indices remained normal, and the animals maintained weight.

Additional data to that shown in Table III suggested that daily doses of 60 and 120 mg. per kilogram may produce a temporary decrease in total hemoglobin and erythrocytes. Further experiments to test this are described in Series 2.

Series 2.—Acetophenetidin 60 and 120 mg. per kilogram. Four dogs received 60 mg. per kilogram of the drug per day for 70 days, and five additional dogs received 120 mg. per kilogram per day for 70 days. The weekly hematologic observations are presented in Fig. 3. During the administration of the 60 mg. per kilogram dose there was a significant decrease in total hemoglobin, erythrocytes, and cell volume within the period of fifteen to forty days, but thereafter these values progressively increased and were not significantly below the control values at the end of the experiment. With the 120 mg. per kilogram

dose, a similar significant decrease in total hemoglobin, erythrocytes, and cell volume occurred during the period of fifteen to fifty days which was followed by some increase in the values. At the end of the experiment some of the values were below the control level, but the average values were not significantly different from the control values. In both groups some increase in reticulocytes

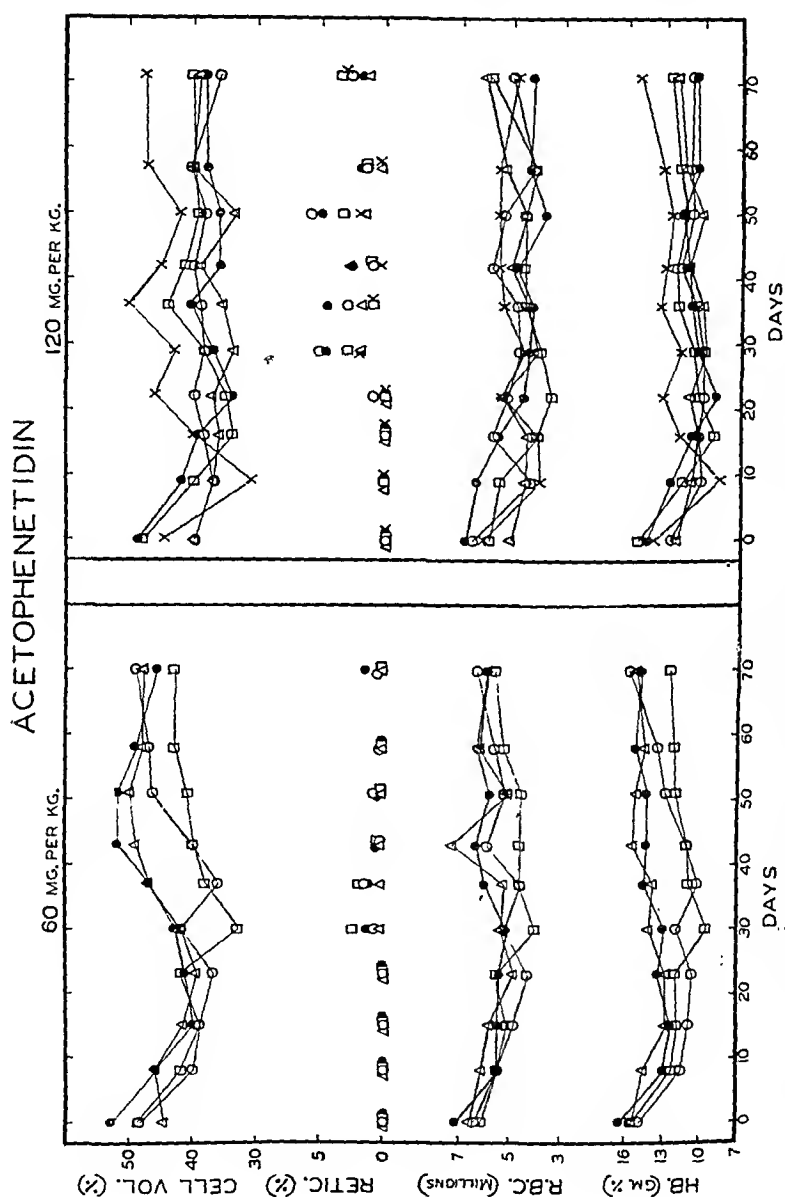


FIG. 3.—Effect of daily administration of acetophenetidin 60 mg. per kilogram (left) and 120 mg. per kilogram (right) on peripheral blood. Each character represents an individual dog.

occurred after twenty-one to twenty-eight days, which was significant in the 120 mg. per kilogram group. The volume and color indices remained within normal limits. No significant change was observed in the total or differential leucocyte counts, and there was no cunulation of methemoglobin or sulfhemoglobin. All animals either maintained or gained weight.

Series 3.—Acetophenetidin in large doses. Acetophenetidin was administered in daily doses of 250 mg. per kilogram for fifteen days to one dog, and for one hundred days to two dogs, and in doses of 200 mg. per kilogram for seventy-six days to two dogs. The two experiments shown in Fig. 4 on two different dogs receiving 250 mg. per kilogram daily for one hundred days are representative. Both experiments demonstrate some effect on the peripheral blood. The total hemoglobin, erythrocytes, and cell volume showed phasic decreases with a

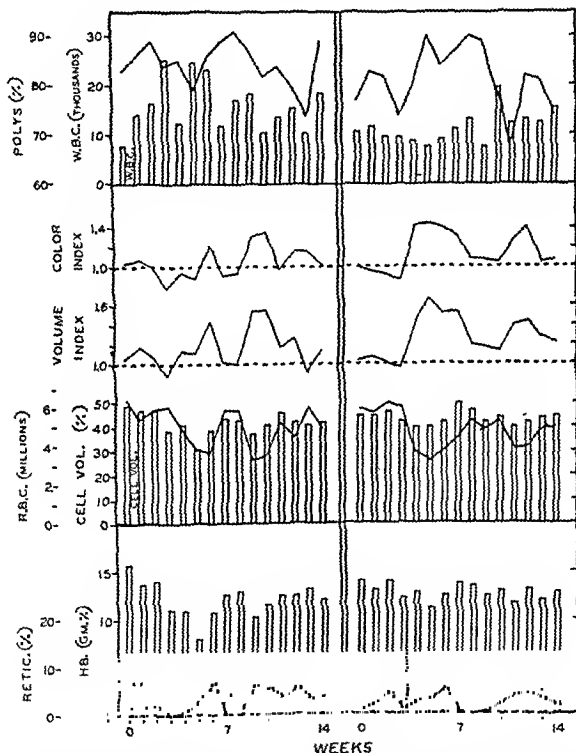


Fig. 4.—Effect of daily administration of acetophenetidin in doses of 250 mg. per kilogram on the peripheral blood of two dogs. The conventions are the same as in Fig. 2 except that time is in weeks.

subsequent trend toward normal values. A decrease in the erythrocyte counts was associated with reticulocytosis. During the phases of anemia there was an increased volume index and a small increase in color index. No consistent effect upon the total or differential leucocyte counts was observed. No cumulation of methemoglobin or sulfhemoglobin occurred. In general the animals maintained or gained weight.

Effect of Acetophenetidin on Bone Marrow.—In Series 1 the counts on femur imprints of the bone marrow from Dogs 26 (15 to 30 mg. per kilogram) and 29 (15, 30, and 60 mg. per kilogram) showed some increase in the polychromatic normoblasts with some decrease in the orthochromatic normoblasts resulting in no significant decrease of the myeloid-erythroid ratio. No change was observed in the femur sections. However, in Dogs P-1 and P-2 in which the dose was increased more rapidly (progressive 15 to 120 mg. per kilogram) the femur imprints showed a marked increase in the pronormoblasts and polychromatic normoblasts; the myeloid-erythroid ratio was significantly decreased to 0.386 and 0.549. Histologic sections of the femur also indicated an increase in percentage of erythroid cells; and the cellularity appeared normal.

The differential rib marrow counts for Series 2 are shown in Table II. With the 60 mg. per kilogram dose the myeloid-erythroid ratio was 0.836 for the rib, and 0.768 for the femur. Some increase in the percentage of pronormoblasts and orthochromatic normoblasts was evident. Femur sections were available on only three animals: two showed some hyperplasia, and the other normal cellularity. For the 120 mg. per kilogram group the average myeloid-erythroid ratios were 0.935 for the rib and 1.029 for the femur. Some increase was evident in the percentage of all the erythroid series except orthochromatic normoblasts. The cellularity of the femur marrow appeared normal in three dogs, and hyperplastic in one. Compared to the effects produced by 60 mg. per kilogram acetophenetidin, the differential counts on the 120 mg. per kilogram group showed a somewhat greater increase in the younger erythroid series, although the total erythroid percentage increase was less. In both cases the myeloid-erythroid ratios were significantly reduced.

Bone marrow studies made on the two dogs of Series 3 shown in Fig. 4 revealed the myeloid-erythroid ratios to be 0.704 and 0.600 for rib smears, and 0.514 and 0.602 for femur imprints. In both cases there was a general increase in the percentage of erythroid cells which was most marked in the polychromatic and orthochromatic normoblasts. Both the white and red series of cells were complete. Histologic sections also revealed some hyperplasia with evidence of increased erythroid activity.

III. METHEMOGLOBIN FORMATION.

It has been stated that with all of the repeated single daily dosages of the drugs employed in this study there was no cumulation of methemoglobin. This has meant that when the blood was examined twenty hours after administration of the drugs either no methemoglobin or only traces were present. A sufficient dose of either acetanilid or acetophenetidin produces a temporary methemoglobinemia in the dog. However, the amount of drug must exceed a certain threshold or minimal dose to produce significant amounts (i.e., 0.4 Gm. per 100 c.c. or twice the error of the method). According to Lester's data,⁶ with which we are in essential agreement, the average threshold dose for acetanilid is about 20 mg. per kilogram, and for acetophenetidin it is a little more than 30 mg. per kilogram. The average maximal methemoglobin formation in dogs with normal total hemoglobin values for the various doses of drugs used in this report have been estimated for the lower range from the data of Lester, and for the higher range from our own data, and are as follows:

ACETANILID		ACETOPHENETIDIN	
DOSE MG./KG.	MHB GM./100 C.C.	DOSE MG./KG.	MHB GM./100 C.C.
9	0.1		
18	0.5	15	0.1
36	1.6	30	0.4
72	4.3	60	1.9
144	5.8	120	3.3
200	6.1	200	4.3

The duration of the methemoglobinemia is related to the size of the dose, but even with the largest doses employed in these studies no more than traces were present after twenty hours, and consequently no cumulation on daily doses was noted. The maximal amount of methemoglobin formed with the larger doses is dependent on the total hemoglobin concentration.²² (A more complete description of our data on methemoglobin formation will appear in a subsequent publication.)

DISCUSSION

The data on acetanilid indicate that the daily administration of 9 or 18 mg. per kilogram doses of the drug produce no significant effect on peripheral blood. Similarly the effects on peripheral blood of 36 mg. per kilogram of the drug daily are probably not significant, although examination of the bone marrow indicates a significant increase in erythropoiesis. The effects upon peripheral blood of 72 mg. per kilogram of acetanilid daily are significant in indicating a tendency toward development of a mild anemia with some reticulocytosis. This observation gains further weight due to the fact that the bone marrow shows a more significant increase in erythroid activity. A moderate degree of anemia is produced by daily doses of 144 mg. per kilogram. The administration of large doses of acetanilid, 250 mg. per kilogram daily for five to ten days, clearly demonstrates the production of a severe hemolytic anemia. When the erythrocytes, total hemoglobin and cell volume decrease, there is a reticulocytosis, an elevation of plasma bilirubin, and a marked increase in erythroid activity in the bone marrow. The appearance of an increased number of normoblasts as well as less mature leucocytes in the peripheral blood also indicates a marked bone marrow response. The anemia is macrocytic with a tendency to be hyperchromic probably due largely to the reticulocytosis. The peripheral blood picture returns rapidly to normal when administration of the drug is stopped. Such experiments have been repeated several times on the same animal and indicate that no depression of marrow activity occurs. The minimal daily dose tested which produced significant effects on peripheral blood was 72 mg. per kilogram.

Acetophenetidin in daily doses of 15 and 30 mg. per kilogram appears to produce no consistent effect upon the peripheral blood. Daily doses of 60 mg. per kilogram cause a mild anemia which is temporary since the peripheral blood picture slowly returns toward normal even with continuation of the drug. At the same time the bone marrow shows a significant increase in erythroid activity. Daily doses of 120 mg. per kilogram produce a slightly greater and somewhat more persistent anemia and a significant reticulocyte response. Although the bone marrow shows evidence of increased erythropoiesis the change is not greater than that observed on the 60 mg. per kilogram dose. However, when the dosage is progressively and rapidly increased to 200 mg. per kilo-

gram there is a greater inversion of the myeloid-erythroid ratio. The continued administration of large doses of acetophenetidin, 250 mg. per kilogram daily for several weeks, does not produce an anemia comparable to that observed with acetanilid. Rather the effect is a phasic decrease in total hemoglobin, erythrocytes, and cell volume followed by recovery. However, the bone marrow shows considerable evidence of increased erythropoiesis. When anemia occurred, it was macrocytic and tended to be hyperchromic. As with acetanilid, the evidence indicates that the anemia is of hemolytic origin.

No significant changes in the total or differential leucocyte counts were observed with either drug except for the leucocytosis during the severe anemia produced by large doses of acetanilid. In the observations on bone marrow, no abnormal cells were seen, and all normal types of cells were present.

Therefore, it appears that in the dog daily doses of these drugs in excess of 36 mg. per kilogram of acetanilid and probably 60 mg. per kilogram of acetophenetidin are required to produce a sustained decrease in the erythrocytes in the peripheral blood. There is no doubt that sufficiently large or toxic doses can produce definite evidence of accelerated erythrocyte destruction as judged by the effects on peripheral blood and responses of the bone marrow. The actions of acetanilid and acetophenetidin are qualitatively similar; however, acetanilid is quantitatively more active in producing a hemolytic anemia. This difference may be due to differences in solubility and absorption, or in the manner in which the drugs are metabolized.

Quantitative comparison of our data on the activity of acetanilid and acetophenetidin in producing hemolytic anemia in the dog with the effects of this activity on man cannot yet be made because of the lack of controlled experiments and bone marrow observations on man. The best approximation, but based only on total hemoglobin, is from the data of Lester¹⁴ which suggests that the dog may be somewhat less susceptible to the development of anemia than man. On the other hand the response of the dog to acetanilid is appreciably greater than that reported for the rat¹⁵ and the monkey,¹ although the qualitative effects on peripheral blood in both species and on the marrow in the monkey appear similar. The hematological actions of acetanilid on peripheral blood in the two dogs reported by Payne⁷ are similar to ours, with the bone marrow showing hyperplasia due to an increase in both erythroblastic and myeloid elements. These bone marrow findings on experimental animals resemble those in man in the only case report found. Lundsteen et al⁴ reported observations on a patient who had taken large doses of both acetanilid and acetophenetidin (daily doses amounting to 1.5 Gm. of each drug); the marrow was hyperplastic with increased leucopoiesis, megakaryopoiesis, and markedly increased normoblastic erythropoiesis.

Both acetanilid and acetophenetidin in sufficient dosage are capable of producing methemoglobinemia in the dog. As is well known, however, methemoglobin is rapidly reconverted to hemoglobin, which explains why we found none 20 hours after administration of the drugs. It is worth noting that no cumulation occurred with repeated single daily doses. The dosage and frequency of dosage causing cumulation in man has been discussed by Lester.¹⁴ We have not observed the formation of significant amounts of sulfhemoglobin with these drugs in the dog, nor any cumulation on chronic administration. Neither acetanilid nor acetophenetidin appear to form methemoglobin directly,

but only after conversion in the body to other substances. It has been suggested that *p*-aminophenol or phenylhydroxylamine may be among the intermediates. (For further discussion and references see 16 and 17.) In our experience *p*-aminophenol is capable of forming methemoglobin directly and rapidly, and is more active in producing anemia than acetanilid or acetophenetidin. This, of course, suggests that if *p*-aminophenol is formed, it can play a definite role in the observed hematological effects. It is quite possible that methemoglobinemia per se may also act as a stimulus to erythropoiesis, since we have shown that a reduced venous oxygen saturation occurs during methemoglobinemia.¹⁴

When sufficiently large doses of these drugs are administered to produce an anemia, our evidence indicates that it is of the peripheral hemolytic type as characterized by reticuloecytosis, increased plasma bilirubin, and hyperplasia of the bone marrow with inversion of the myeloid-erythroid ratio. The peripheral blood picture rapidly returns to normal when the administration of the drugs is stopped. In acute experiments, splenectomy did not appear to influence the development of the anemia with acetanilid nor the speed of recovery. We have been able to reproduce an almost identical peripheral blood-and-bone marrow picture by chronic bleeding alone.

No definite conclusion has yet been reached concerning the exact mechanism of the hemolytic action of acetanilid and acetophenetidin. These drugs do not appear to be direct hemolysins in ordinary concentrations. However, preliminary experiments indicate that they can accelerate saponin lysis of washed red cells (method of Ponder¹⁵). It is not unlikely that the postulated metabolic breakdown products, *p*-aminophenol and phenylhydroxylamine, may also be concerned in the hemolytic action. The fact that acetanilid and acetophenetidin can produce methemoglobinemia could easily lead to the speculation that this action may be related to the process of hemolysis. Brownlee²⁰ has reported increased urinary porphyrin excretion in rats following administration of acetanilid and acetophenetidin. The increased porphyrin excretion was identified as due largely to coproporphyrin III. It has been suggested that this represents an abnormal pathway for the degradation of hemoglobin, possibly through methemoglobin.^{20, 21} Rimington²¹ has noted a correlation between the action of aromatic amino compounds to increase porphyrin excretion and to form methemoglobin. However, increased porphyrin excretion did not follow sodium nitrite administration. We have also noted some correlation between the activities of a series of aromatic amino compounds to produce methemoglobinemia and hemolytic anemia. The data of this report also indicate that the doses of acetanilid and acetophenetidin producing definite hemolytic effects exceed the threshold dose for methemoglobin formation. In spite of these inferences, however, and on the basis of work now in progress, it is our view that the ability of a drug to produce methemoglobinemia and hemolytic anemia may be only coincident actions dependent on certain physical and chemical properties. At least the production of methemoglobin per se cannot be accepted as a part of the hemolytic process without more direct proof.

SUMMARY

1. The hematological effects of a wide range of dosages of acetanilid and acetophenetidin on the peripheral blood and bone marrow of dogs are described.

2. The chronic administration of acetanilid up to 36 mg. per kilogram per day caused little or no abnormal hematological changes in dogs.

3. Large doses of acetanilid produce a hemolytic anemia with no evidence of depression of bone marrow. Recovery is rapid and apparently complete on cessation of administration of the drug.

4. The daily administration of acetophenetidin produces the same qualitative effects, but quantitatively the drug is less active than acetanilid.

5. Both drugs produce a temporary methemoglobinemia when certain threshold doses are exceeded. However, in the dosage range employed, no cumulation of methemoglobin was observed with repeated daily administration.

6. The possible mechanisms of the hematological actions are discussed.

The authors are grateful to Dr. J. L. Selwind of the Department of Anatomy for many helpful suggestions.

REFERENCES

1. Smith, P. K.: Prolonged Administration of Large Doses of Acetanilid in Monkeys With Special Reference to Blood Changes, *J. Pharmacol. & Exper. Therap.* 68: 1, 1940.
2. von Oettingen, W. F.: The Aromatic Amino and Nitro Compounds, Their Toxicity and Potential Dangers, *Public Health Bulletin N* 941.
3. Hanzlik, P. J.: Health Hazards in Acetanilid- and Mixtures, *J. Am. Dent. A.* 27: 1505, 1672, 1833, 1940.
4. Lundsteen, E., Meulengraecht, E., and Rischel, A.: Chronic Acetanilid Poisoning, *Acta Med. Scandinav.* 96: 462, 1938.
5. Knaeke, R. R.: Relation of Drug Therapy to Neutropenic States, *J. A. M. A.* 111: 1255, 1938.
6. Lester, D.: Formation of Methemoglobin. I. Species Differences With Acetanilide and Acetophenetidine, *J. Pharmacol. & Exper. Therap.* 77: 154, 1943.
7. Payne, S.: Acetanilid Poisoning. A Clinical and Experimental Study, *J. Pharmacol. & Exper. Therap.* 53: 401, 1935.
8. Heller, V. G., and Paul, H.: Changes in Cell Volume Produced by Varying Concentrations of Different Anticoagulants, *J. Lab. & Clin. Med.* 19: 777, 1933-34.
9. Van Loon, E. J., and Clark, B. B.: Hematology of the Peripheral Blood and Bone Marrow of the Dog, *J. Lab. & Clin. Med.* 28: 1575, 1943.
10. Todd, J. C., and Sanford, A. H.: Clinical Diagnosis by Laboratory Methods, page 322, ed. 8, Philadelphia, 1937, W. B. Saunders Co.
11. Evelyn, K. A., and Malloy, H. T.: Microdetermination of Oxyhemoglobin, Methemoglobin and Sulfhemoglobin in a Single Sample of Blood, *J. Biol. Chem.* 126: 655, 1938.
12. Malloy, H. T., and Evelyn, K. A.: The Determination of Bilirubin With the Photoelectric Colorimeter, *J. Biol. Chem.* 119: 481, 1937.
13. Fisher, R. A., and Yates, F.: Statistical Tables for Biological, Agricultural and Medical Research, ed. 2, London, 1943, Oliver and Boyd.
14. Lester, D.: Formation of Methemoglobin. II. Repeated Administration of Acetanilide and Acetophenetidine, *J. Pharmacol. & Exper. Therap.* 77: 160, 1943.
15. Smith, P. K., and Hambourger, W. E.: Effects of Acetanilid on the Growth and Blood Morphology of Rats, *J. Pharmacol. & Exper. Therap.* 57: 34, 1936.
16. Bernheim, F.: The Interaction of Drugs and Cell Catalysts, Minneapolis, 1942, Burgess Publishing Co.
17. Clark, B. B., Van Loon, E. J., and Morrissey, R. W.: Acute Experimental Aniline Intoxication, *J. Indust. Hyg. & Toxicol.* 25: 1, 1943.
18. Clark, B. B., Van Loon, E. J., and Adams, W. L.: Respiratory and Circulatory Responses to Acute Methemoglobinemia Produced by Aniline, *Am. J. Physiol.* 139: 64, 1943.
19. Ponder, E.: Acceleration of Haemolysis in Relation to Chemical Structure. I. Benzene Derivatives, *J. Exper. Biol.* 16: 38, 1939.
20. Brownlee, G.: The Role of the Aromatic Amino Group in Deranged Pigment Metabolism, *Biochem. J.* 33: 697, 1939.
21. Rimington, C., and Hemmings, A. W.: CXVIII. Porphyrinuric Action of Drugs Related to Sulphanilamide. Comparison With Reported Toxicity, Therapeutic Efficacy and Causation of Methaemoglobinemia. Definition of the Structure Responsible for Porphyrinuric Action, *Biochem. J.* 33: 960, 1939.
22. Lolli, G., Lester, D., and Rubin, M.: Formation of Methemoglobin. III. The Influence of Total Hemoglobin on the Formation of Methemoglobin From Acetanilide, *J. Pharmacol. & Exper. Therap.* 80: 74, 1944.

THE EFFECT OF ESTRONE ON ANAEROBIC GLYCOLYSIS OF THE UTERUS OF THE RAT IN VITRO

BEATRICE M. SWEENEY, PH.D., ROCHESTER, MINN.

THE study of the estrogens has reached a stage at which their gross effects on organs need to be understood in terms of a fundamental change brought about by these substances in the biochemistry of a single cell. Such an understanding of the basic mechanism by which estrogens act not only would be of theoretic interest but would facilitate greatly the rational therapeutic use of these substances. A number of studies have been made with this in mind, among these a series of investigations on the effect of estrogens on the respiration of various tissues, particularly uterine tissue. In the following report, I shall deal only with anaerobic glycolysis, since it has been found that this process usually showed a greater and more rapid change than did the aerobic respiration in animals treated with estrogens.

Kerly¹ reported that the rate at which the uterus of the rat in vitro produced lactic acid was doubled during proestrus, as compared with that at diestrus or in uteri from spayed animals. She² showed that the rate of anaerobic glycolysis of uteri from spayed rats into which 2.5 micrograms of estrone in 1 per cent alcohol had been injected twenty-four to thirty-six hours previously was double that of uteri from rats into which estrone had not been injected. When estrone was added to the uterine tissue in vitro, however, no effect of 0.1, 2 or 25 micrograms of estrone was observed. However, her observations on the effect of estrogens in vitro were complicated by the side effects of the solvent.

Carroll^{3, 4} reported a study of the effects that injection of large amounts (50 micrograms) of estradiol-2-propionate produced on anaerobic glycolysis of uteri of spayed rats. He also found that uteri taken from animals into which this estrogen had been injected twenty-four hours before produced lactic acid anaerobically at a higher rate than did uteri from spayed controls into which estradiol-2-propionate had not been injected, although the difference was not as great as that reported by Kerly. Endometrium and muscle of the uterus were studied separately and stimulation occurred in both.

The purpose of this study was to investigate further the effect of estrone added to uterine tissue in vitro under anaerobic conditions and to determine if possible whether the increased rate of anaerobic glycolysis of uteri from rats treated with estrogens is a primary effect of estrogens on the cells of the uterus or merely a result of their otherwise stimulated condition.

METHOD

Rats.—The uterine tissue used in these experiments was taken from white rats spayed at the age of three months. The sensitivity of the rats to estrone

From the Division of Experimental Medicine, Mayo Foundation.
Received for publication, April 24, 1944.

was standardized in the following way: following spaying, rats were rested one week, then smeared one week to determine whether spaying was complete. If these rats showed absence of estrous cycles, 1 microgram of estrone in 1 c.c. of olive oil then was injected, according to the usual assay procedure. Only rats positive for this dose were used. Following a week's rest 0.5 microgram of estrone was injected into these rats and only those negative for this dose were retained. The rats were used for experiments during the following week, the fifth after spaying.

Preparation of tissue.—Rats were killed by a blow on the head. The uterus was dissected out immediately, slit lengthwise and placed in warm phosphate buffer of pH 7.4. One horn of each uterus was used as a control while the substance to be tested was added to the other horn.

Bicarbonate Ringer's solution of the following composition:

96 parts NaCl	0.15 M.
2 parts KCl	0.15 M.
2 parts CaCl_2	0.15 M.
20 parts NaHCO_3	0.15 M. (Just acid to phenolphthalein)
2.4 parts Glucose	10 per cent

was measured into the experimental flasks from a storage vessel containing an atmosphere of 95 per cent of nitrogen and 5 per cent of carbon dioxide.

Dilutions of estrone* were made up in bicarbonate Ringer's solution, 0.1 microgram in each cubic centimeter or less as a solution, higher concentrations as a fine suspension.† All solutions were made up with double distilled water, the second distillation having been carried out in a glass still. The pH of all solutions was checked at the beginning and end of each experiment with a Beckman glass electrode. The pH of the solutions used was 7.1 to 7.3. The activity of the solutions of estrone was checked by bio-assay.

Tissues were weighed quickly on a Roller-Smith torsion balance and placed in the experimental flasks. The flasks were attached to their manometers and placed in the thermostatically controlled water bath at $38 \pm 0.1^\circ \text{C}$. A gas mixture of 95 per cent of nitrogen and 5 per cent of carbon dioxide, freed from oxygen by being passed over hot reduced copper wire and saturated with water vapor, was passed through the air space in the experimental vessels for ten minutes. Vessels then were closed and readings made in the usual way⁵ every half hour. The $Q_{\text{G}}^{\text{N}_2}$ (the rate of anaerobic production of lactic acid expressed as cubic millimeters of carbon dioxide formed per hour per milligram dry weight of tissue) was calculated. Values for $Q_{\text{G}}^{\text{N}_2}$ in the tables are averages of these rates for each half hour.

Direct determinations of the total lactic acid produced during the experiment were made by the method of Barker and Summerson.^{6†}

*Crystalline estrone was donated by Parke, Davis and Company.

†I wish to express my gratitude to Dr. H. L. Mason, who made up all estrone solutions and suspensions.

‡I wish to express my indebtedness to Dr. J. L. Bollman and Dr. E. V. Flock, under whom this work was carried out, for their valued suggestions. Technical assistance was kindly given by Evelyn Cunningham, Henrietta Schasch, Cecily Barker and Emery Van Hook.

RESULTS

The average rate of anaerobic glycolysis of untreated uteri from spayed rats was 7.0 ± 1.4 , a value comparable to that obtained by other workers for uteri of spayed rats (Kerly,¹ 11; Kerly,² 7; Carroll,^{3,4} for endometrium only, 6.1). The rate of production of lactic acid did not show any tendency to fall off during the four to six hours of the experiments. Production of lactic acid determined by the direct method of Barker and Summerson⁵ at the end of the experiments averaged 23.9 ± 2.3 micrograms per milligram of tissue per hour, which is in reasonably close agreement with the lactic acid calculated from the total carbon dioxide evolved from bicarbonate, 23.4 ± 4.7 micrograms per milligram of tissue per hour. There was no evidence in any experiment to suggest that any substance other than lactic acid was liberating carbon dioxide during anaerobic glycolysis.

When 5 micrograms of estrone in 1 c.c. of bicarbonate Ringer's solution were injected into rats twenty-four hours before the removal of the uterus,

TABLE I

THE RATE OF ANAEROBIC GLYCOLYSIS ($Q_0^{N_2}$) OF UTERINE TISSUE OF SPAYED RATS IN DIFFERENT CONCENTRATIONS OF ESTRONE, IN VITRO

UNINJECTED RATS					INJECTED RATS	
WITHOUT ESTRONE	WITH ESTRONE, MICROGRAMS PER CUBIC CENTIMETER				WITHOUT ESTRONE	WITH ESTRONE, 0.01 MICROGRAM PER CUBIC CENTIMETER
	5	0.1	0.01	0.001		
5.8					6.3	
5.3					8.4	
6.6					10.5	9.0
6.6					8.6	8.7
6.6					10.8	10.9
8.3					11.6	
8.3					10.4	
4.8	6.1					
6.1	6.5					
7.7	7.2					
4.6	4.4					
-	6.0					
-	6.1					
-	3.9					
-	7.7					
6.3		5.1				
6.3		3.9	6.3			
6.0			5.6			
8.7			7.0			
5.1				6.2		
Mean						
$6.4 \pm 0.3^*$	6.0 ± 0.5	4.5 ± 0.6	6.5 ± 0.6	--	9.5 ± 0.7	9.5 ± 0.7

23.

23

*The numbers after the plus or minus signs are standard errors of the respective means. These are calculated by the formula $S.E. = \sqrt{\frac{\sum(x-\bar{x})^2}{n(n-1)}}$ in which x represents the successive variates in the sample, \bar{x} the mean of the sample and n the number of variates.

the $Q \frac{N_2}{G}$ of the uterus was about 35 per cent higher than in similar tissue from spayed controls, the difference tending to be greater during the first few hours of the experiments than later. However, the $Q \frac{N_2}{G}$ of uteri treated in vitro with a number of concentrations of estrone (Table I) was not significantly different from that of untreated uteri, even at the end of six hours.

Decrease of sensitivity of the uterine cells to estrone during the fifteen to thirty minutes necessary for dissection and preparation of tissue probably does not account for the absence of effect of estrone in vitro, since injection of 0.1 and 0.01 microgram directly into the uterus before dissection was also ineffective.

It was thought possible that the failure of estrone to increase the $Q \frac{N_2}{G}$ in vitro was caused by the absence of some necessary supplementary factor. A number of substances, succinate, malate, indole-acetic acid and standard chorionic gonadotropin, were added in the presence of estrone (Table II), but no such effect could be observed. If a supplementary substance exists, it was thought possible that this might be present in rat plasma. No evidence for

TABLE II

THE EFFECT OF SUBSTANCES OTHER THAN ESTRONE WITH AND WITHOUT ESTRONE ADDED IN VITRO ON $Q \frac{N_2}{G}$ OF UTERINE TISSUE OF SPAYED RATS

CONTROL UTERI			UTERI WITH OTHER SUBSTANCES				
WITHOUT ESTRONE	WITH ESTRONE		SUBSTANCE	CONCENTRATION	WITHOUT ESTRONE	WITH ESTRONE	
$Q \frac{N_2}{G}$	$Q \frac{N_2}{G}$	CONCENTRATION, MICROGRAMS, PER C.C.			$Q \frac{N_2}{G}$	$Q \frac{N_2}{G}$	
3.4	3.9	5	Succinate	.0001 M	6.1	5.8	
4.8	6.1	5		.0001 M	8.0	7.2	
7.0, 7.3	—	0.01	Malate	.001 M	9.1	9.2	
6.1	6.5	5	Indole-3-acetic acid	10/mg./1	6.7	5.2	
7.4	3.9, 5.1	5	Standard chorionic gonadotropin	10 I.U./c.c.	6.4	6.1, 5.7	
6.6		5		10 I.U./c.c.	6.0		
6.3		0.1		10 I.U./c.c.	—	3.4	
8.7		0.01		0.1 I.U./c.c.	2.6	4.3, 6.1	
6.0		0.01		0.1 I.U./c.c.	4.9	7.5	
5.1	6.2	0.001		0.1 I.U./c.c.	7.0	6.6	
7.4	5.1, 3.9	0.1		0.01 I.U./c.c.	8.0	2.8	
6.3				0.01 I.U./c.c.	—		
7.4				0.001 I.U./c.c.	6.4		
		0.01	Plasma of uninjected rats	3 c.c.	8.0	6.8	
		0.01			13.1	7.7	
			Plasma of rats injected with 5 micrograms estrone	3 c.c.	8.4	9.1	
		0.01					
		0.01	Plasma of rats injected with 50 micrograms estrone	3 c.c.	11.0	8.6	

the presence of such a factor in plasma from rats in which estrone had not been injected or from rats into which large doses of estrone had been injected twenty-four hours before was obtained.

Carroll^{3,4} found that, when tissues were weighed at the end of an experiment, considerable error could be introduced through loss of particles of tissue into the medium. However, he used much smaller amounts of tissue than those used in these studies. If the absolute values for $Q_G^{N_2}$ are perhaps too high because of this error, there proved to be no difference in the percentage dry weight between treated and control uteri, 18.8 per cent as compared with 18.9 per cent.

The rate of anaerobic respiration of uterine tissue either with or without estrone showed great variability. This would seem to be inherent in the tissue rather than in the method of measurement since the determinations of lactic acid agreed fairly well with manometric readings. Small variation of experimental conditions may be at fault, also. This large variation possibly may obscure a small effect of estrone *in vitro*, in no way comparable to the large increase of the rate of production of lactic acid in uteri from animals given estrone by injection.

COMMENT

Since estrone in any concentration used failed to increase the $Q_G^{N_2}$ of uterine tissue when treated *in vitro*, it seemed likely that the increase of $Q_G^{N_2}$ which was observed after injection of estrone into the whole animal either must depend on the presence in the tissues of some accessory substance brought there as a result of the injected estrone or must be due to a change of the nature of estrone itself or of the uterine cells which does not occur *in vitro* and probably requires time. That accessory substances necessary for action of estrone on the uterus are not present in the plasma of uninjected or injected rats was shown. Furthermore, experiments by Emmens⁷ which show that estrone in minute amounts produces estrus in spayed animals when injected directly into the vagina, suggest that it is not necessary for estrone to enter the circulation to bring about its effects. This makes the necessity for the release of accessory substances from tissues other than the uterus itself unlikely.

It would seem, then, that either estrone as such is inactive on uterine tissue or that the changes of the rate of anaerobic glycolysis observed after injection of estrone are the result of some other change or series of changes in the cells of the uterus and cannot be assigned a primary role in the mechanism of action of estrogen.

SUMMARY

A series of concentrations of estrone in bicarbonate Ringer's solution was found not to have any effect on the rate of anaerobic glycolysis of uterine tissue of spayed rats *in vitro*. The lack of effect of estrone was not changed by the presence of a number of different substances.

REFERENCES

1. Kerly, Margaret: The Effect of the Oestrous Cycle on the Metabolism of Isolated Rat Uterus, *J. Biochem.* 31: 1544, 1937.
2. Kerly, Margaret: The Effect of Oestrone on the Metabolism of Rat Uterus, *J. Biochem.* 34: 814, 1940.
3. Carroll, W. R.: The Influence of Estrogens on the Metabolism of the Uterus, Thesis, Cambridge, Massachusetts, 1942.
4. Carroll, W. R.: Influence of Estrogens on Respiration of Rat Uterine Tissue, *Proc. Soc. Exper. Biol. & Med.* 49: 50-52, 1942.
5. Dixon, Malcolm: Manometric Methods as Applied to the Measurement of Cell Respiration and Other Processes, Cambridge, 1934, The University Press, 122 pp.
6. Barker, S. B., and Summerson, W. H.: The Colorimetric Determination of Lactic Acid in Biological Material, *J. Biol. Chem.* 138: 535, 1941.
7. Emmens, C. W.: Precursors of Oestrogens, *J. Endocrinol.* 2: 444, 1941.

CLINICAL CHEMISTRY

STUDIES ON THE INGESTION OF LARGE QUANTITIES OF PROTEIN AND AMINO ACIDS

ALFRED H. FREE, PH.D., AND JACK R. LEONARDS, PH.D., CLEVELAND, OHIO

THE role of proteins in the diet has been appreciated and stressed for a long period of time. Rubner¹ some forty years ago pointed out that man cannot live on lean meat alone because of the limitation of the apparatus of mastication. This same idea is maintained in current textbooks of physiology^{2, 3} which suggest that the limitations are due to those of digestion and absorption. It is appreciated that certain Eskimo tribes may subsist for long periods on diets of meat and fish, but in these foods the fat component is relatively high. Recently the effect of large quantities of protein in the digestive tract has received attention by reason of the fact that following massive gastrointestinal hemorrhage there is a considerable increase in the urea nitrogen content of the blood.^{4, 5}

The question of the superiority of amino acid mixtures over protein in the dietary management of certain pathologic conditions has attracted considerable attention. There is some feeling that orally administered amino acid mixtures might be superior to similar quantities of dietary protein,^{7, 8} although the actual demonstration of such a superiority has not been made.

The present report describes studies carried out in two normal human subjects in whom comparable massive amounts of whole blood proteins, muscle proteins and amino acid mixtures were ingested orally. One objective of these studies was to compare the efficiency of the gastrointestinal tract in handling protein and in handling amino acids. A second objective was to get a better understanding of the effect of massive amounts of digested protein on blood and urine urea and blood amino acids. It was felt that the effect of massive gastrointestinal hemorrhage on blood urea could better be understood and evaluated with such information available.

METHODS

The experiments reported here consisted essentially of studying the changes in blood and urine during and following the ingestion of large amounts of meat, blood, and amino acids. The amount of nitrogen consumed in each case was of the same order of magnitude. This quantity represented the maximum

From the Department of Biochemistry, School of Medicine, Western Reserve University, Cleveland, Ohio.

Supported by a grant from the Willams-Waterman Fund of the Research Corporation, New York.

Received for publication, June 1, 1941

amount of meat that could be voluntarily consumed over a period of approximately eight hours. The blood and amino acids were ingested over a comparable time interval. The meat was ground lean horse meat and was baked without addition of any other materials. The fresh whole beef blood was obtained from a local packing house and was immediately defibrinated and chilled. In this form it was quite palatable and was ingested in the same manner as one would drink milk. The solution of amino acids represented a casein hydrolysate fortified with tryptophane.* This solution was taken orally in the same way as the blood. The amount of nitrogen in each of the materials was determined by chemical analysis. The authors served as the experimental subjects. Their weights were 80 kg. (F) and 75 kg. (L). The meals on the day before each experiment were chosen so as to contain a minimum of protein, and in addition no food was consumed for eighteen hours prior to, and eighteen hours following, the experiment. The experiments were conducted about two weeks apart.

A blood sample was obtained in the fasting state and at 1, 3, 5, 8, 11, 16, 24, 33, and 48 hours after the beginning of the experiment. The blood was allowed to clot and the serum was analyzed for amino acid nitrogen, urea, and protein. A control urine sample was obtained for a three-hour period before starting the ingestion of the food materials and at frequent intervals for 48 hours thereafter. Urine samples were analyzed for urea, and the maximum urea clearance was calculated for each interval. The determination of urea in blood and urine was carried out by the colorimetric method described by Ormsby.⁹ Serum amino acid nitrogen was determined by the colorimetric method of Folin as modified by Danielson.¹⁰ This method was adapted to the use of the Evelyn photoelectric colorimeter by simple dilution of the final color. Plasma protein and all total nitrogen determinations were carried out by micro-Kjeldahl distillation procedures.

RESULTS

The maximum amounts of protein which each subject could ingest as ground baked meat were 480 grams (F) and 320 grams (L). This amount of food, representing $5\frac{1}{4}$ and $3\frac{3}{4}$ pounds of fresh meat, respectively, ingested over eight hours produced some gastrointestinal discomfort characterized by a sense of abdominal fullness. One subject (F) was able to ingest the same amount of protein in the form of blood (2,250 c.c. of whole blood) although the general abdominal discomfort produced by the blood exceeded that obtained with the meat. The second subject was able to ingest only 85 per cent as much protein in the form of blood as in the form of meat over the same time interval. Both subjects readily agreed that the amino acid mixture was much more disagreeable than either the meat or blood. One subject (L) had a severe attack of vomiting midway in this experiment so that total intake of amino acids was about half that of meat protein. The other subject was able to ingest a quantity of amino acid nitrogen equal to that ingested as blood or meat, but regards the experience as an extremely disagreeable ordeal. The gastrointestinal distress resulting in both subjects from amino acid ingestion

*Frederick Stearns & Co., Detroit, Mich.

was not due to the taste of this mixture which many persons do not like. A slight amount of diarrhea was obtained in one of the subjects (L) following blood and amino acids, but not in the other subject although prevention of a bowel movement required considerable voluntary control. A considerable diuresis was obtained in all of the experiments.

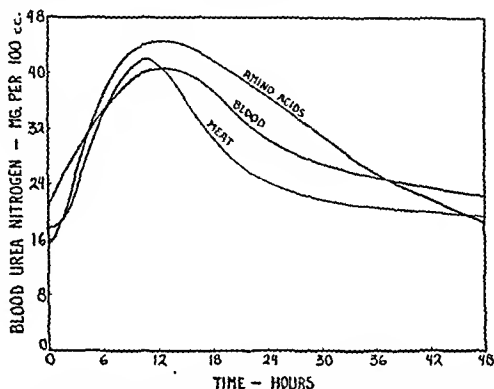


Fig. 1.—Changes in blood urea nitrogen, following the oral ingestion of meat, blood, or amino acids. Data on subject F who ingested 480 grams of protein or amino acid mixture.

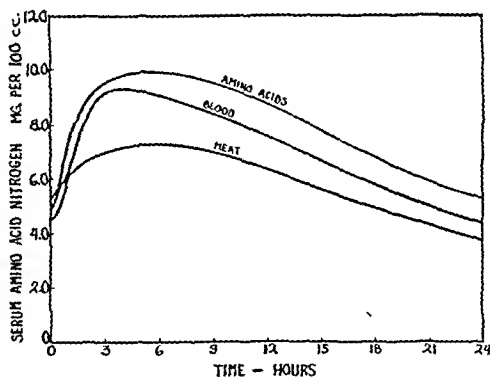


Fig. 2.—Changes in serum amino acid nitrogen following the oral ingestion of meat, blood, or amino acids. Data on subject F who ingested 480 grams of protein or amino acid mixture.

The changes in blood urea nitrogen and blood amino acid nitrogen for subject F obtained following the ingestion of blood, meat, and amino acids are indicated in Figs. 1 and 2. It will be seen that a comparable increase in blood amino acid nitrogen occurred following the ingestion of each of the three test substances although there was a lesser rise with the meat. The changes in

blood urea nitrogen were also comparable following the ingestion of blood, meat, or amino acids, with the amino acid mixture causing a slightly greater increase in blood urea nitrogen. The urinary urea nitrogen excretion in this subject is indicated in Table I. The ingestion of large amounts of proteins or amino acids caused a great increase in urea output in the urine. Calculations of the urea clearance indicate that there was a considerable increase in urea clearance during the height of the urea excretion in all three experiments. The total urea nitrogen excretion for forty-eight hours following the ingestion of the 76.8 Gm. of nitrogen in the form of meat, blood, and amino acids was 59.4 Gm., 72.1 Gm., and 71.1 Gm., respectively. Thus it appears as though most of the nitrogen derived from the proteins ingested was excreted as urea in the first forty-eight hours.

TABLE I
UREA EXCRETION AND UREA CLEARANCES FOLLOWING THE ORAL INGESTION OF
MEAT, BLOOD, OR AMINO ACIDS

TIME INTERVAL HOURS	URINE UREA NITROGEN EXCRETION GRAMS PER HOUR			UREA CLEARANCE C.C. PER MINUTE		
	MEAT	BLOOD	AMINO ACIDS	MEAT	BLOOD	AMINO ACIDS
-3 - 0	0.78	0.93	0.72	77	73	79
0 - 2	0.66	1.05	0.62	65	75	59
2 - 4	0.80	1.25	0.94	62	76	61
4 - 8	1.56	2.20	1.43	81	112	69
8 - 11	2.30	2.18	2.43	97	103	95
11 - 16	2.17	2.35	2.62	92	105	100
16 - 24	1.47	1.70	1.99	75	82	83
24 - 33	0.92	1.18	1.27	60	70	67
33 - 48	0.78	1.09	0.97	64	74	70

The plasma proteins were determined at intervals following the ingestion of amino acids. The values obtained were 6.8, 7.0, 6.9 and 7.1 Gm. per 100 c.c. at 0, 11, 33 and 48 hours, respectively. This does not represent any significant change. Electrophoretic studies of the serum proteins were also carried out to determine whether any of the protein fractions were changed. No changes were found.*

The results of the studies obtained on the other subject (L) were similar to those that have been presented for subject (F). The changes in amino acid nitrogen and blood urea nitrogen level of the blood were almost identical in the two subjects for the three test substances, the meat giving a lesser rise in serum amino acid nitrogen in both subjects. The blood urea nitrogen increase following amino acid ingestion was somewhat less than that obtained in subject (F) undoubtedly due to the fact that only about one-half of the amount of the amino acid nitrogen was ingested. Maximum urea clearances were increased with all three test substances and almost all of the ingested nitrogen was excreted as urea nitrogen in the urine.

DISCUSSION

The authors agree with the statements made in texts of physiology which suggest that a man cannot readily meet his daily calorie requirement by the ingestion of lean meat. The fact that hydrolyzed amino acids cannot be in-

*The electrophoretic studies were carried out by Lt. C. L. SanClemente, to whom the authors are deeply grateful.

gested in quantities exceeding that of protein indicates that the limiting factor is not one of mastication as Rubner¹ originally suggested. Neither is it one of digestion, since the amino acids do not require digestion, but rather it is the rate of intestinal absorption that defines the maximum intake of protein or amino acids. The high osmotic pressure of the amino acid mixture undoubtedly greatly retards its gastric emptying. The authors have observed that a quantity of glycine as small as 50 Gm. is only very slowly emptied from the human stomach, 50 per cent of this quantity remaining at the end of three hours. In three subjects this amount of glycine caused considerable nausea and gastrointestinal distress of the same nature as that obtained with the amino acid mixture.

The blood urea clearance figures indicate that there is an actual increase in the maximum clearance as well as in the amount of urea excreted. These results are in accordance with the observations of Jolliffe and Smith¹¹ on dogs and Longley and Miller¹² in human beings, in which it was found that high levels of protein intake elevated urea clearance values. Study of the data in Table I indicates that the rate of urea excretion is from 2.5 to 3.6 times as great during the time that the blood urea level is increased. This great increase in the rate of excretion of urea by the normal kidney is quite important in minimizing the extent of blood urea elevation. In an individual with some impairment of renal function it is apparent that, if the kidney could not increase its rate of urea excretion, the blood urea level would show a much greater rise. The same effect would tend to be observed following massive gastrointestinal hemorrhage where the functional efficiency of the kidney is decreased as a result of diminished blood pressure. The mechanism of the rise in blood urea following digestion of large amounts of protein or following severe hemorrhage is undoubtedly different from that observed by Myers¹³ in patients with chronic peptic ulcer.

The value for the blood amino acid nitrogen levels, for the blood urea nitrogen levels, and especially for the urea excretion and the maximum amount of the substance that could voluntarily be ingested indicate that the over-all processes of digestion, absorption, and metabolism of muscle proteins as compared with blood proteins are approximately the same. Furthermore the data obtained following the ingestion of the amino acid mixture provide further evidence that the controlling or limiting factor is the rate of intestinal absorption. These results raise a definite question as to the efficacy of orally administered amino acids and their possible superiority over dietary protein. If there is an inadequacy of pancreatic proteinases to accomplish the hydrolysis of dietary protein, then orally administered amino acids might be of advantage. On the other hand, the osmotic pressure of amino acid mixtures is many times as great as that of protein and for this reason they have an irritating effect on the gastrointestinal tract. From the experience with normal subjects it would appear that under almost all circumstances protein would be more readily tolerated by the gastrointestinal tract than amino acids. Obviously the utility of intravenously administered amino acids is in no way concerned in this discussion.

One purpose of this investigation was to determine whether or not the azotemia following extensive gastrointestinal hemorrhage may be used to

evaluate the extent and duration of the bleeding. Attempts have been made to utilize the blood urea nitrogen levels as diagnostic and prognostic aids.^{6, 14-16} The value of such attempts has also been disputed.¹⁷ In one experiment described in this report 76 Gm. of protein nitrogen in the form of blood were ingested, but never as much as 3 per cent of this nitrogen appeared in the blood at any one time as increased blood urea nitrogen. If one assumes equal distribution of urea in all of the body water the quantity of increased urea nitrogen is always less than 20 per cent of the nitrogen ingested. It is readily apparent that the increase in blood urea nitrogen is influenced by factors other than the amount of blood in the gastrointestinal tract. These may include differences in the rates of digestion, absorption and metabolism of protein as well as differences in renal efficiency in different normal subjects. The latter factor would be even more important in patients following extensive hemorrhage due to variable decreases in blood pressure. Thus it would appear as though the measurement of the blood urea nitrogen level is an extremely crude method of ascertaining the extent of gastrointestinal bleeding, since the extent of elevation is a function both of the amount of protein digested and of the rate of urea excretion. The latter factor is subject to wide variations.

The data on urine urea nitrogen excretion in Table I indicate that the urea excretion is a good index of the amount of protein metabolized, particularly if some attention is also directed toward blood urea levels. It is readily admitted that any blood protein that is lost by vomiting or by diarrhea will not be detected by studies of the urea content of urine. The extent of hemorrhage, however, will be much more accurately evaluated by urine and blood studies than by blood studies alone. Sufficient information can be obtained if two complete 24-hour urine collections are made and the total urea nitrogen is determined. This figure multiplied by the factor 6.25 will give an index of the amount of protein metabolized, particularly if it is known that the blood urea has not changed greatly during this period. Corrections for any increase in blood urea nitrogen are readily calculated assuming that urea is equally distributed in all of the body water. From the amount of protein metabolized it is possible to calculate the amount of blood that would have to be digested to supply this quantity of protein. Deductions should be made for any dietary protein ingested during the period. It would appear that this method, although subject to certain limitations, gives a more reliable estimate of the extent of a gastrointestinal hemorrhage than any of the methods in use at the present time.

SUMMARY AND CONCLUSIONS

1. Maximum amounts of blood, muscle meat, and amino acid mixture were ingested over an eight-hour period by two normal human subjects. Studies of blood urea, blood amino acids, urinary urea excretion, and urea clearances were carried out.

2. The rate of intestinal absorption appears to be the limiting factor in determining the amount of protein or amino acid mixture that can be ingested. The normal gastrointestinal tract more readily handles large amounts of unhydrolyzed protein, than it does equivalent amounts of amino acids. This raises a question as to whether amino acids have any advantage in alimentation

over unhydrolyzed dietary protein, especially when relatively large amounts are administered and when pancreatic proteinases are present in adequate amounts.

3. The variations in the blood levels of urea nitrogen and amino acid nitrogen were similar after the ingestion of the two proteins and the amino acid mixture.

4. Maximum urea clearances, as well as urinary urea excretion, were increased following the consumption of amino acids and proteins.

5. The extent of a gastrointestinal hemorrhage can be more accurately evaluated by urine and blood urea determinations than by blood studies alone.

REFERENCES

1. Rubner, M.: *E. von Leyden's Handbook der Ernährungstherapie*, Leipzig, 1: 42, 1903.
2. Best, C. H., and Taylor, N. B.: *The Physiological Basis of Medical Practice*, ed. 3, Baltimore, 1943, Williams & Wilkins Company, p. 928.
3. Gemmill, C. L.: *Macleod's Physiology in Modern Medicine*, ed. 9, St. Louis, 1941, The C. V. Mosby Company, p. 791.
4. Christiansen, T.: Biochemical Changes in the Organism Produced by Massive Intraintestinal Hemorrhage, *Rev. Gastroenterol.* 4: 166, 1937.
5. Schiff, L., and Stevens, R. J.: Elevation of Urea Nitrogen Content of the Blood Following Hematemesis or Melena, *Arch. Int. Med.* 64: 1239, 1939.
6. Chunn, C. F., Harkins, H. N., and Boals, R. T.: Alimentary Azotemia and the Bleeding Peptic Ulcer Syndrome, *Arch. Surg.* 43: 773, 1941.
7. Elman, R.: *Protein Metabolism and the Practice of Medicine*, M. Clin. North America 27: 303, 1942.
8. Boling, C. A., and Lee, R. E.: Treatment of Hypoproteinemia by Oral Administration of Protein Hydrolysate, *Arch. Surg.* 43: 735, 1941.
9. Ormsby, A. A.: A Direct Chlorimetric Method for the Determination of Urea in Blood and Urine, *J. Biol. Chem.* 146: 595, 1942.
10. Danielson, I. S.: Amino Acid Nitrogen in Blood and Its Determination, *J. Biol. Chem.* 101: 505, 1933.
11. Jolliffe, N., and Smith, H. W.: The Excretion of Urea in the Dog. II. The Urea and Creatinine Clearance on Cracker Meal Diet, *Am. J. Physiol.* 99: 101, 1931.
12. Longley, L. P., and Miller, M.: The Effect of Diet and Meals on the Maximum Urea Clearance, *Am. J. M. Sc.* 203: 253, 1942.
13. Myers, V. C.: *The Practical Chemical Analysis of Blood*, ed. 2, St. Louis, 1924, The C. V. Mosby Company, p. 35.
14. Schiff, L., Stevens, R. J., and Moss, H. K.: The Prognostic Significance of the Blood Urea Nitrogen Following Hematemesis or Melena, *Am. J. Digest. Dis. & Nutrition* 9: 110, 1935.
15. Ingegnio, A. P.: The Elevated Blood Urea of Acute Gastro-Intestinal Hemorrhage and Its Significance, *Am. J. M. Sc.* 190: 770, 1935.
16. Kaump, D. H., and Parson, J. C.: Extrarenal Azotemia in Gastrointestinal Hemorrhage. (II.) Experimental Observations, *Am. J. Digest. Dis.* 7: 191, 1940.
17. Johnson, J. B.: The Pathogenesis of Azotemia in Hemorrhage From the Upper Gastrointestinal Tract, *J. Clin. Investigation* 20: 161, 1941.

LABORATORY METHODS

GENERAL

THE SIGNIFICANCE OF THE pH IN THE GOLD REACTION

CARL LANGE, M.D., AND ALBERT H. HARRIS, M.D., ALBANY, N. Y.

THE satisfactory performance of tests that represent modifications of the original gold reaction¹ requires an understanding of the effect of the pH of the milieu on the character of the curves. If the pH is outside the relatively narrow optimum range, the results of tests will be vitiated. At the time that Zsigmondy published his work on the so-called "gold number,"² and when the original paper concerning the gold reaction appeared in 1912, the pH was not then taken into consideration. No trouble attributable to the pH, however, was encountered so long as the original technique employing Zsigmondy's formol gold was followed, because, fortunately, the pH happened to be in the optimum range. Early modifications aimed at technical simplification involved replacement of the formol gold, since the preparation was claimed to be too difficult for clinical laboratories. This difficulty, encountered by many laboratory workers in the preparation of formol gold, was actually the only safeguard for the maintenance of an adequate pH of the milieu in the original gold reaction, as batches of colloidal gold satisfactory in appearance were certain to be properly adjusted with regard to the pH. When the formol gold was replaced by modifications, these safeguards were lost.

Now that the significance of the pH in the gold reaction is understood the use of gold sols of varying pH is possible, provided the milieu in which the reaction takes place is properly adjusted. The variations in the pH of the three reagents taking part in the reaction—cerebrospinal fluid, diluent, and gold sol—and the effect on the pH of the milieu will be analyzed first.

THE pH OF EACH OF THE THREE REAGENTS

The pH of freshly collected cerebrospinal fluid is 7.4, that is, almost neutral. Unless loss of carbon dioxide is prevented, the pH rises markedly in a few hours. The pH of mailed cerebrospinal fluid is usually about 8.4 to 8.6, a reaction sufficiently alkaline to disturb the pH of the milieu.

The pH of the diluent is of far greater importance, since this reagent can be made to stabilize the reaction in the optimum range. In the original gold reaction, 0.4 per cent saline was used in preparing the serial dilutions of cere-

From the Division of Laboratories and Research, New York State Department of Health, Albany.

Received for publication, April 7, 1944.

cerebrospinal fluid. The pH of saline is about 5.7 to 6.0, a markedly acid reaction, due to absorption of carbon dioxide from the air. The pH of serum diluted with saline remains unchanged in the lower dilutions, since the highly concentrated protein acts as a buffer, while in higher dilutions the low pH of the saline exerts an effect. On the other hand, this buffering effect is negligible in cerebrospinal fluids normally containing only traces of protein and the pH may vary in serial dilutions from 8.6 to 5.7.

Reducing agents other than formol have been used in the preparation of gold sols, and have yielded hydrogen-ion concentrations differing markedly from the gold sols prepared with formol. Since the reduction with sodium citrate introduced by Borowskaja² is the most satisfactory, discussion will be confined to this type. The sensitivity and appearance of citrate gold sols vary widely, depending on the concentration of the alkaline sodium citrate used to reduce the acid gold chloride. Stable gold sols can be prepared with sodium citrate concentrations varying from 1:1,000 to 1:15,000. With decreasing concentrations of the alkaline sodium citrate there is, of course, a decrease in the pH, while the sensitivity of the gold sol increases. There is no sharply restricted optimum pH for the reduction. The reaction, however, must be acid, since no reduction occurs at or above the neutral point. Borowskaja used a high concentration of the sodium citrate, namely 1:2,000. The resulting gold sol, which for convenience may be called "citrate gold 1:2,000," is extremely "hard," that is, insensitive. The pH is about 6.0 by potentiometric determination. Saline is used as a diluent.

In the quantitative gold reaction, of which a detailed description has been given in a previous publication,⁴ "citrate gold 1:10,000" is used, the pH of which is, of course, even lower, namely 4.8 to 5.0. This "softer" citrate gold, however, exhibits the same optimal sensitivity as formol gold. The marked acidity is of no consequence, because the final pH of the milieu is regulated by buffers.

The optimal sensitivity of gold sols is directly dependent not only on the dispersity of the sol itself but also on the pH of the milieu. If a gold sol that is too hard, such as that of Borowskaja, is used in a milieu of correct pH, that is, 7.4, the results are inferior because the sol is too insensitive. If, on the other hand, the sensitivity of a gold sol that is too hard is raised by a pH of the milieu that is too low, the results of the gold reaction may be rendered definitely false, as indicated by the occurrence of false parietic curves subsequently discussed. In short, only through a combination of an optimal sensitivity and an optimum pH of the milieu can best results be reproduced.

EXPERIMENTAL DETERMINATION OF THE OPTIMAL pH OF THE MILIEU

The optimal pH of the milieu depends to some extent on the sensitivity of the gold sol. It is determined in the following experiments on the basis of a sensitivity believed to be optimal, and that, in addition, can be reproduced without difficulty. The technique of the quantitative gold reaction⁴ is employed. The term quantitative is applied since the principle of the reaction involves the use of a gold sol of constant and optimal sensitivity and the maintenance of a constant and optimal pH of the milieu through strong phosphate buffers. In

TABLE I
EFFECT OF INADEQUATE pH ON RESULTS OF GOLD REACTION

NO.	MATERIAL	TOTAL PROTEIN MICRO- KJELDAHL MG. %	PHOS- PHATE BUFFERS pH	GOLD REACTION—SERIAL DILUTIONS*										SUM TOTAL†	TYPE OF GOLD CURVE
				1:15	1:23	1:34	1:51	1:76	1:114	1:171	1:256	1:384	1:576		
1	Purified sheep hemoglobin solution	43.40	7.2	0	0	0	0	0	2	2	3	1.5	1.5	10	
2			7.0	20	20	20	18	18	17	17	17	15	9	171	
3	Normal cerebrospinal fluid	19.4	7.4	2.5	3	3.5	4.5	4	3.5	3	2.5	1.5	1	29	Typical normal
4	The same normal cerebrospinal fluid to which was added 1:100 volume of hemolyzed human blood	207	7.4	2	2.5	2	2	2	2	2	2	2	2	21	Atypical curve in subnormal range
5			6.5	18	18	18	18	18	18	18	18	18	18	180	Strong parietic
6	Cerebrospinal fluid from meningococcus meningitis	108	7.4	3	3	3.5	4	5	6	8	9	11	8.5	61	"Meningitic"†
7			6.2	18	18	18	18	17	17	16	16	16	16	170	Atypical parietic

*Note changed serial dilutions, begin with 1:15, common factor 3/4, instead of 1/2.

†Sum of the 10 color values read with color standard; expresses strength of reaction. Values range from 0 red to 10 blue, to 20 colorless.

^ marks the maximum reaction obtained by reading after two hours.

†"Meningitic" now called "hematogenous type" indicating the passage of blood colloids into the cerebrospinal fluid.

these experiments presented in Table I, buffers of varying pH but of uniform molecular strength were used.

Citrate gold 1:10,000 was employed because through its use the reproduction of correct gold sols was remarkably easy. In order to eliminate the deleterious effect of aging, use of this gold sol was restricted to a period of one week. There were no disadvantages in following this precaution, because the gold sol is not only easily prepared, but is exceedingly inexpensive. This technique, which can be readily adopted in any competent laboratory, was followed in these experiments (see Table I). The results were read with the help of an easily prepared gold color standard and were in this way objectively recorded.

A few readily duplicated experiments suffice to demonstrate the detrimental effect of an excessively low pH on the results of the gold reaction. Experiments 1 and 2 show the striking effect of such a slight change of the pH as from 7.2 to 7.0, on the reaction between gold sol and purified hemoglobin solution, the isoelectric point of which lies at about 6.8 to 7.0. No. 3 demonstrates the reaction of a normal cerebrospinal fluid in the quantitative gold reaction, while Nos. 4 and 5 show the reaction of the same cerebrospinal fluid after the addition of 1:100 volume of hemolyzed human blood. When the milieu is adjusted to pH 7.4, the blood has a weakening effect, while at pH 6.5 a fundamental change in the character of the curve appears, No. 5 is an example of a "false paretic curve." It exactly counterfeits a true paretic curve, but it is technically false, and can be easily avoided by the maintenance of an adequate pH. This is of great practical importance in the examination of fluids containing small amounts of blood that have been accidentally introduced during the tap and have become hemolyzed. No. 6 exemplifies the typical "shift to the right" or so-called "meningitic curve" occurring at a pH of 7.4 with cerebrospinal fluid from a case of meningitis. No. 7 shows the effect of a low pH, 6.2, on the same cerebrospinal fluid. In this case, even in the absence of hemoglobin with its high isoelectric point, occurrence of the most dangerous type of false reaction is demonstrated. It is obvious that the value of a paretic curve becomes nil if, of all types of the gold reaction, this most important one is polluted with a considerable percentage of technically false paretic curves. The appearance of paretic curves in purulent meningitis constitutes the same among false reactions since, of the protein patterns indicated by the distinguishable types of the gold reaction, those encountered in paresis and in purulent meningitis exhibit in every respect the maximal difference.

It has thus been demonstrated that false paretic curves may be elicited by a pH that is too low. The higher the pH of the milieu, the greater the safeguard against "false paretic curves." Above pH 7.4, however, the strength of the reaction decreases unfavorably. Accordingly, pH 7.4 was selected as optimum. It is safely above pH 7.0, the danger zone in which hemoglobin may render the results of the colloidal gold reaction entirely misleading. It is noteworthy that this pH, 7.4, is optimum for the reduction of Zsigmondy's formol gold sol.

DISCUSSION

The pH of the milieu has been shown to be of great importance. The pH of the cerebrospinal fluid and gold sol is significant only in systems in which the

pH of the milieu is not adjusted, as is the case when saline is used as a diluent. When strong buffers are employed, the pH can be fixed at the optimum value, regardless of the particular pH values of the cerebrospinal fluid and gold sol. Modifications of the original gold reaction that do not include a satisfactory regulation of the pH can be shown to yield erroneous results. The obtaining of a so-called paretic type of curve in the examination of a specimen from a case of purulent meningitis is evidence that the technique employed is worse than valueless. Experiments described in this paper indicate that such results may be elicited by a pH that is too low, and experience has shown that they occur only when the pH is unsatisfactory. In more than thirty years, using at first the original gold reaction, which is only a qualitative test, and later the quantitative gold reaction,⁴ the senior author has never observed anything suggesting a paretic type of curve in cases of purulent meningitis. The regulatory mechanisms governing the pH in these two techniques have been explained. The pH of the milieu in the case of the original gold reaction was not fixed and constant, since buffers were not used; however, the pH usually remained in a range high enough to assure good results. When too low, the appearance of the sol was obviously wrong. The quantitative gold reaction requires buffers to maintain a constant pH at the optimum level. The pH in modifications of the gold reaction in which it is not taken into consideration is usually incorrect, either owing to the use of gold sols that are themselves acid, or to the acidifying of gold sols in order to increase their sensitivity to a satisfactory level.⁵ The combination of inherently acid gold sols and a saline diluent leads to erroneous results; the magnitude of error is restricted, however, since the fall in pH is limited. Error in the highest degree may be encountered in all modifications in which acid is added to gold sols in order to increase their sensitivity; under such circumstances the pH of the milieu is certain to be inadmissibly low. While inherently acid gold sols may yield optimal results in combination with buffers that insure the correct pH; buffers cannot be used for this purpose when gold sols are acidified according to the Krebs principle, since adequate sensitivity of the inadequate gold sol is obtained only by adjusting the reaction at a pH far below the optimum for the test itself. The fact that satisfactory results cannot be obtained under such circumstances is convincingly demonstrated by the occurrence of "false paretic curves."

SUMMARY

Optimal and reproducible results of the gold reaction depend to a large extent on a constant and optimal pH of the milieu. The alkalinity of cerebrospinal fluids or the acidity of gold sols can affect the pH of the milieu unfavorably only if saline is used as a diluent. Strong buffers are superior in every respect because they serve to maintain a uniform and correct pH. The optimal pH of the gold reaction is 7.4, provided that an easily prepared citrate gold sol of constant and optimal sensitivity is used. A pH of the milieu that is too low causes erroneous results. An inadmissibly low pH may easily be detected in unsatisfactory modifications of the gold reaction by the appearance of false paretic curves in purulent meningitis.

REFERENCES

1. Lange, Carl: Die Ausflockung kolloidalen Goldes durch Cerebrospinalflüssigkeit bei infectiösen Affektionen des Zentralnervensystems, *Ztschr. f. Chemother.* 1: 44-78, 1912.
2. Schulz, Fr. N., and Zsigmondy, R.: Die Goldzahl und ihre Verwertbarkeit zur Charakterisierung von Eiweißstoffen, *Beit. z. chem. Phys. u. Path.* 3: 137-160, 1902.
3. Borowskaja, D. P.: Zur Methodik der Goldsolbereitung, *Ztschr. f. Immunitäts.* 82: 178-182, 1934.
4. Lange, Carl: Methods for the Examination of Spinal Fluid, *Am. J. Syph., Gonorr., & Ven. Dis.* 23: 638-668, 1939, (Technique of "Quantitative Gold Reaction," pp. 654-662).
5. Krebs, H. A.: Zur Goldsolreaktion im Liquor cerebrospinalis, *Klin. Wchnschr.* 4: 1309-1312, 1925.

A RAPID AGGLUTINATION TEST FOR THE DIAGNOSIS OF TULAREMIA

SAMUEL R. DAMON, PH.D., AND MARY B. JOHNSON, B.S., MONTGOMERY, ALA.

THE diagnostic laboratory, in setting up routine agglutination tests, is not infrequently requested to include in the series the *Pasteurella tularensis* antigen. The results of such tests are generally read and reported after an incubation period of 18 to 24 hours, although it would be an advantage in many cases if the report to the physician could be made earlier.

In our experience it has been observed that definitive results with the *P. tularensis* antigen can be obtained almost immediately. This is accomplished by setting up the routine series of serum dilutions—in this laboratory $\frac{1}{40}$, $\frac{1}{80}$, $\frac{1}{160}$, and $\frac{1}{320}$ —and adding the same antigen used in the test tube agglutination. The tubes are then shaken vigorously, by hand or in a Kahn shaker, for four or five minutes and read.

For the sake of comparison we have recorded the immediate results of the "shake test" and parallel incubated tests after the usual 18 to 24 hours. Thus far the degree of reaction observed has been identical, not only in the routine dilutions but in the final titer of the serum, except in a few instances where a slightly stronger reading has been obtained following the overnight incubation,

TABLE I

P. TULARENSIS AGGLUTINATION OBTAINED BY THE RAPID AND CONVENTIONAL METHODS

SPEC. NO.	SHAKE TEST				TITER	WATER BATH TEST				TITER
	$\frac{1}{40}$	$\frac{1}{80}$	$\frac{1}{160}$	$\frac{1}{320}$		$\frac{1}{46}$	$\frac{1}{80}$	$\frac{1}{160}$	$\frac{1}{320}$	
75	4+	4+	4+	4+	4+ $\frac{1}{320}$	4+	4+	4+	4+	4+ $\frac{1}{320}$
106	4+	4+	4+	4+	4+ $\frac{1}{2560}$	4+	4+	4+	4+	4+ $\frac{1}{2560}$
162	4+	4+	4+	4+	4+ $\frac{1}{2560}$	4+	4+	4+	4+	4+ $\frac{1}{2560}$
279	4+	4+	4+	4+	4+ $\frac{1}{2560}$	4+	4+	4+	4+	4+ $\frac{1}{2560}$
499	3+	4+	4+	4+	Not deter- mined	4+	4+	4+	4+	Not deter- mined
585	3+	2+	—	—		4+	2+	—	—	
3436	4+	3+	1+	—		4+	3+	—	—	
C. C.	4+	4+	4+	4+	4+ $\frac{1}{640}$	4+	4+	4+	4+	4+ $\frac{1}{640}$
R. G.	4+	4+	4+	4+	4+ $\frac{1}{5120}$	4+	4+	4+	4+	4+ $\frac{1}{5120}$
Dog 1644	4+	4+	4+	4+	4+ $\frac{1}{1280}$	4+	4+	4+	4+	4+ $\frac{1}{1280}$
S.S.	4+	4+	4+	4+	4+ $\frac{1}{2560}$	4+	4+	4+	4+	4+ $\frac{1}{2560}$

From the Bureau of Laboratories, State Department of Public Health.
Received for publication, May 11, 1944.

e.g., a 3+ has become 4+. In no instance has any degree of reaction been obtained in an overnight test when the results of the "shake test" were completely negative.

Table I gives typical results obtained by both procedures, together with the final titers of the sera. All the specimens were from human sources except one, which was from an experimentally infected dog.

CONCLUSION

Reliable agglutination results in suspected cases of tularemia may be obtained almost immediately by the technique described.

A SIMPLE INEXPENSIVE APPARATUS FOR THE STERILE LYOPHILIZATION OF SMALL AMOUNTS OF MATERIAL

CARL U. DERNEHL, M.D.,* MADERO N. BADER, D.V.M.,* GALVESTON, TEXAS,
AND LUIS H. BARTLETT, M.S. (ENG.),† AUSTIN, TEXAS

THE Department of Preventive Medicine and Public Health of the University of Texas has been conducting researches on Rickettsial diseases for the past four years; this work has required the use of a large number of laboratory animals. In the past, all strains of our passages were kept in guinea pigs and rats. Because of the expense involved, however, another method was sought for preserving these strains. It seemed desirable to try lyophilizing tissues, but we did not feel justified in purchasing a lyophilic apparatus. We developed, therefore, a setup capable of drying small amounts of material which is simple in design and construction; its cost is negligible.

The apparatus is made entirely of Pyrex glass so that cleaning and sterilization may be done with dry heat. The design decided upon requires only the simplest of glass blowing and offers no great difficulties in construction. With it, from 2 to 5 c.c. of material can be dried in three to five hours' time, depending on the amount of tissue left in suspension. The material to be dried is ground in a mortar; alundum is used whenever necessary. Sterile skimmed milk is added and the whole is thoroughly mixed. Two to five cubic centimeters of this mixture is put into the drying tube and placed in the lyophilic apparatus. When alundum is used, the material is centrifuged or strained through four thicknesses of gauze before it is put into tubes.

The apparatus is illustrated in Fig. 1. Its assembly requires (1) a 500 ml. round bottom flask with three 24/40 standard taper necks; (2) three 24/40 standard taper inner connections with one of them having an inner extension; (3) a Pyrex glass stopcock; (4) a 29/42 standard taper inner and outer connection; (5) a straight walled Pyrex vacuum bottle having a diameter of 15 cm. and a depth of 25 cm.; (6) a laboratory pump supplying adequate vacuum; and (7) a standard drying tower.

CONSTRUCTION

The inner 29/42 connection is sealed to one end of a piece of 25 mm. Pyrex glass tubing 20 cm. long, and the 24/40 connection with the inner extension is sealed to the other end. The inner connection is cut off 3 cm. from the ground glass joint so that it will project a short distance into the flask when the apparatus is assembled. The glass tubing is bent to form a 70 degree angle in such a way that there are no constrictions to obstruct the free flow of air. The outer 29/42 connection is then sealed off at its distal end so

*From the Department of Preventive Medicine and Public Health, University of Texas, Medical Branch, Galveston, Texas.

†From the Bureau of Engineering Research, University of Texas, Austin, Texas.

Received for publication, June 9, 1944.

that it forms a cup 13 cm. deep. A 15 cm. test tube when placed in this cup extends a short distance up into the inner 29/42 connection when the two are joined.

A short piece of Pyrex glass tubing is then sealed onto another 24/40 inner connection and bent at a right angle to act as a connection to the vacuum pump. A Pyrex glass stopcock is sealed to the end of another 24/40 inner connection and serves as a means of attaching a manometer to the system and as a means of releasing the vacuum when so desired.

The connection to the pump is made through a short length of heavy walled rubber pressure tubing having an inner diameter no less than the 6 mm. opening of the vacuum pump. The connection to the manometer is also made with rubber pressure tubing of small bore.

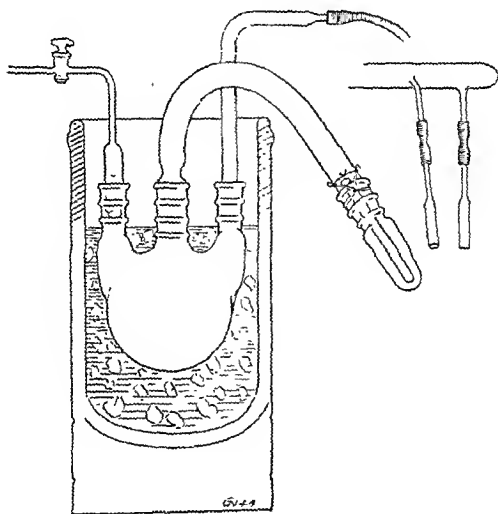


Fig. 1

OPERATION

After the application of a thin layer of stopcock grease to all joints, the three 24/40 inner connections are put into place in the 500 ml. flask; the joint having the inner extension is placed in the center neck. The rubber connections are made and the flask is placed in the vacuum bottle and packed in dry ice. The material to be dried is placed in a 15 cm. test tube with a 12 mm. diameter and is frozen to the walls by being rotated at an angle in a dry ice-acetone freezing mixture. The tube is then placed at once in the cup formed by the 29/42 connection and attached to the apparatus. The pump is started and drying is begun.

After an adequate drying period, the pump is shut off and air which has been passed through both a drying tower packed with calcium sulfate and through a sterile cotton filter, is slowly allowed to enter the system through the stopcock. After the vacuum is released, the test tube is removed from the apparatus and closed immediately with a close fitting sterile rubber vial cap. A sterile needle, attached to the vacuum pump through a length of rubber tubing, is put through the rubber cap and the tube is again evacuated. The needle is rapidly withdrawn and the stoppered end of the tube is plunged at once into melted paraffin to seal any possible leaks.

Subsequent re-solution of the material in the same sterile container without contamination from outside air can be accomplished by simply injecting through the rubber stopper any desired amount of diluent. If small amounts of the dry material are removed, the rest may be preserved if a fresh sterile cap is used and evacuation is done as before.

The utility of the apparatus is increased if a second manifold, which allows the use of ampules, is made in the following way: A 20 cm. piece of 20 mm. Pyrex tubing is sealed to a 24/40 standard taper joint having an inner extension. The inner extension is cut off as before and a right angle bend is made in the tubing. The end of the tube is sealed off and two side arms of 8 mm. Pyrex tubing are sealed into the manifold to allow for the attachment of two ampules. The manifold is placed in the center neck of the 500 ml. flask and the ampules are attached by means of a short length of close fitting pressure tubing. After dehydration is complete, the ampules are sealed off by means of a gas-oxygen flame.

Since difficulty was experienced in sealing the ampules while evacuated, it was decided to fill the ampules with nitrogen gas. This was accomplished by filling a spirometer of sufficient volume with nitrogen and attaching the spirometer to the lyophilization apparatus by a length of rubber tubing leading to the stopcock in the 500 ml. flask. A layer of mineral oil on top of the water in the spirometer keeps the gas fairly dry. The stopcock is then opened slightly and the gas is slowly allowed to replace the vacuum. When the gas passes through the flask, any water present is removed. After the vacuum is neutralized, the ampules can be sealed without any difficulty.

SUMMARY

The construction and operation of an apparatus suitable for the sterile lyophilization of small amounts of material is described.

AN IMPROVED RESTRAINING DEVICE FOR HEMATOLOGICAL WORK WITH RATS AND MICE

G. C. CASPER, B A., ROCHESTER, N. Y.

IN OUR laboratories considerable difficulty has been experienced in restraining small animals like rats and mice for routine blood examination. Various devices such as special boxes, glass test tubes, wrapping in towels, etc., have been used in the past. These methods have in general proved cumbersome or time consuming and some did not have sufficient flexibility to accommodate animals of different sizes.

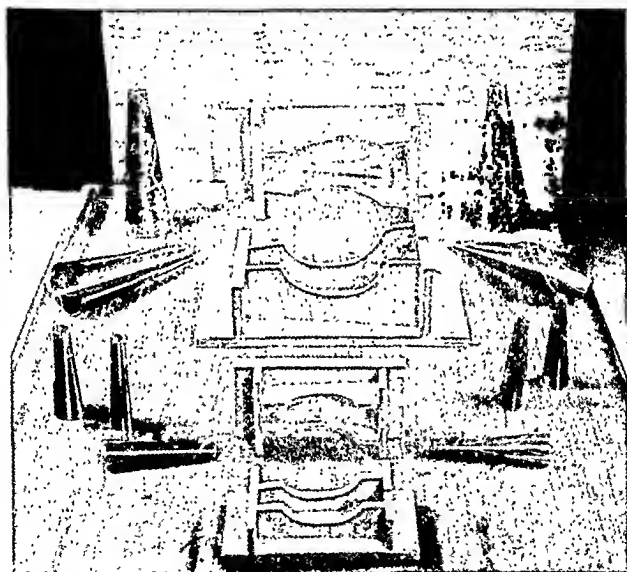


FIG. 1.—Two rat boxes and two mouse boxes are shown, as well as a number of cones of various sizes

Because of the rather large number of rats and mice involved in our work, the necessity for an improved method of restraining the animals became acute. Therefore a number of cones of cellulose acetate (washed x-ray films) were wound on a tapered wooden form having the large diameter sufficient to ac-

From the Department of Radiology, School of Medicine and Dentistry, University of Rochester, Rochester, N. Y.

Received for publication, May 20, 1941.

commodate the largest rat used, with the taper approximately the same as the average rat and the smaller open end of such a size that the animal's head could not emerge. A number of cones of various diameters were assembled to allow some selection for animals of different sizes. Transparent scotch tape proved to be more satisfactory for holding the cones together than did cements which were first tried. Experience soon showed that a length of about 7 inches was sufficient to hold any laboratory rat in our stock. A shallow, open topped

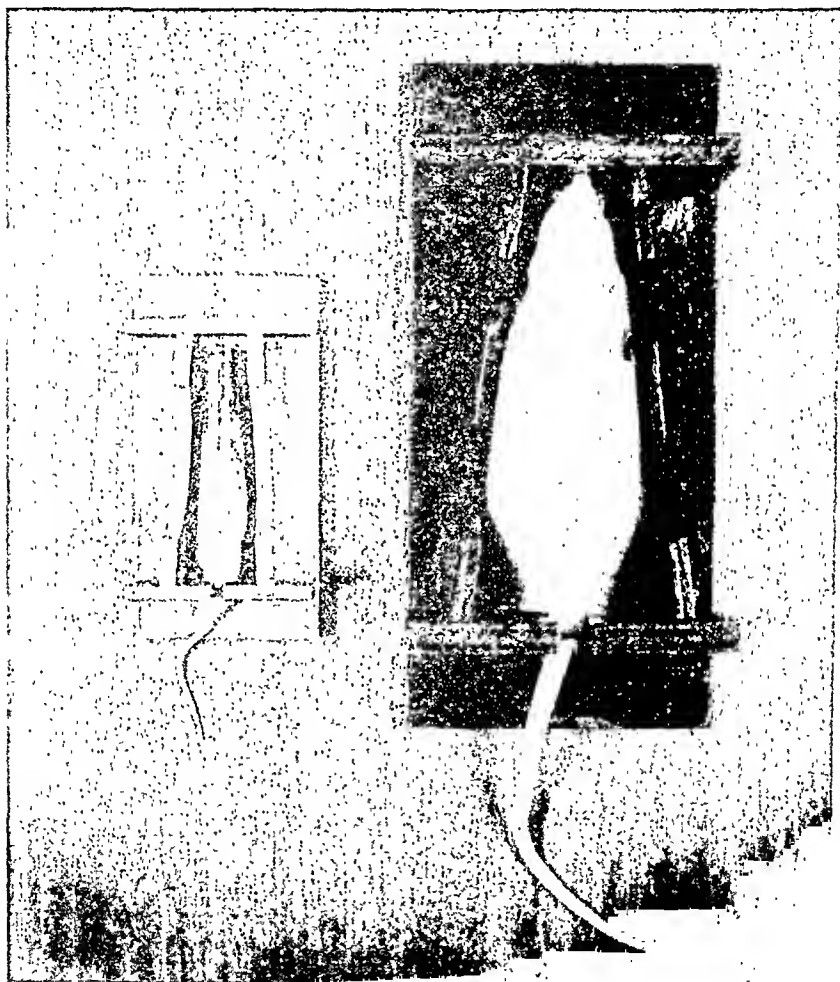


Fig. 2.—Rat and mouse boxes loaded for work.

wooden box (Fig. 1) was constructed with the sides set at the angle necessary to guide and support the plastic cones described above. Wooden cradles were tacked to the ends of the box on the inside to hold the cones more or less level. A slit was made on the back of the box to allow the tail of the animal to hang out (Fig. 2). The end of the tail could then be snipped with a seissors to obtain blood for counts, films, etc. The arrangement constructed for mice is similar except that the boxes and cones are built to a scale suitable to the smaller animal.

Most of the animals enter the cone willingly when it is presented; once they enter, the cone is tipped so that the animal has to climb. Then the loaded cone is placed in the wooden holder and counts are made. The boxes are covered with a waterproof paint so that they are easily cleaned with soap and water.

A simple holder has been described which greatly facilitates the handling of small animals such as rats and mice for hematological work. One great advantage is that the technician can perform the whole procedure without assistance.

A CANNULA FOR MEASURING INTRATHORACIC PRESSURE

K. G. WAKIM, M.D., AND W. D. GATCH, M.D., INDIANAPOLIS, IND.

IT IS sometimes important to measure the intrathoracic pressure and follow the changes in its magnitude during both inspiration and expiration under various experimental conditions. The apparatus and method described below yield uniform results. The apparatus is simple and can be used continuously during an experiment. The cannula can be introduced repeatedly, because of its small caliber, without leaving a leak in the thoracic wall at the end of several observation periods. We have used it on dogs and obtained very satisfactory results.

The Y-shaped cannula, shown assembled in the top diagram of Fig. 1, consists of the following parts: a trocar in the form of a needle control valve (*a*), knurled packing control (*b*), rubber packing (*g*), housing for needle control valve (*c*), knurled adjustment nut (*d*), and a sponge rubber washer (*e*). The portion of the cannula that is introduced through the thoracic wall into the pleural cavity is about one inch in length and one-sixteenth of an inch in diameter. In the bottom diagram of Fig. 1, the sectional view of the cannula demonstrates its connection with the intrathoracic cavity at the black arrows. The white arrows demonstrate the continuity of the intrapleural space with the lumen of the apparatus and rubber tubing (*f*), which connects with a water manometer not shown in this diagram. The U-shaped water manometer to be used with this apparatus should have long arms in order to provide for ample excursions of negative and positive variations in intrathoracic pressure. The water in the manometer may be colored with a blue or red dye to facilitate accurate reading of the pressures.

The shaft of the cannula with the tip of the trocar in its lumen is introduced into the intrapleural cavity through an intercostal space, preferably close to the upper border of a rib near the middle of the lateral wall of the thorax. The depth can be regulated by the use of one or more sponge rubber washers (*e*), and by the knurled adjustment nut (*d*), both of which are anchored to the chest wall by four skin sutures as seen on the anterior view of the knurled nut. After introduction of the shaft of the cannula into the pleural space, the trocar is pulled out as far as the grooved mark, thus establishing an airtight communication between the thoracic cavity and the manometer. A T-connection should be placed in the tube leading to the manometer to provide a means for introducing 50 c.c. of air into the pleural space to facilitate registration of the intrathoracic pressure. Several lateral perforations are made in the part of the

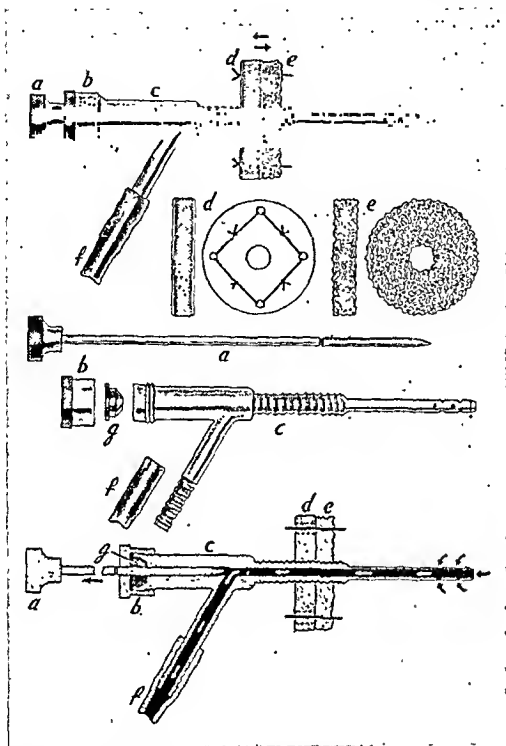


Fig. 1.—The top diagram shows the Y-shaped cannula assembled. The middle diagram shows the constituent parts, and the lower diagram gives a sectional view of the cannula when it is in operation for recording intrathoracic pressure. (Drawing made by J. F. Glore.)

cannula which is introduced into the pleural cavity in order to maintain continuity between the thoracic cavity and the manometer which otherwise might be interrupted by contact of the long surface with the terminal hole during the expansion of the lungs.

A MODIFIED PIPETTE FOR PROTHROMBIN DETERMINATIONS

F. L. MUNRO, PH.D., PHILADELPHIA, PA.

WE HAVE frequently encountered difficulty in pipetting small amounts of reagents into test tubes such as are used in the Quick method for prothrombin determination. If the tip of the pipette is placed near the top of the test tube, the reagent has to run down the length of the tube, with the result that a considerable part of the 0.1 c.c. of reagent used is lost. On the other hand, if the tip of the pipette is placed near the bottom of the test tube, there is a tendency for the reagent to form a film between the pipette and the test tube wall, again resulting in the loss of material.

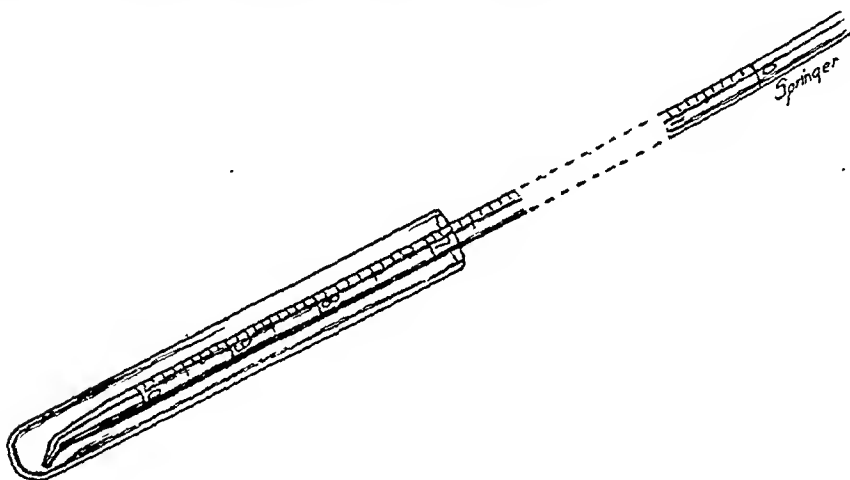


Fig. 1.

We have found that these difficulties may be overcome by drawing out and bending the tip of the pipette as shown in the diagram. With a pipette so modified, the tip may be placed near the bottom of the test tube without the formation of a film and the loss of fluid is negligible.

CHEMICAL

LABORATORY TESTS OF THE OXIMETER WITH AUTOMATIC COMPENSATION FOR VASOMOTOR CHANGES

MAJOR ALLAN HEMINGWAY,* AIR CORPS, A.U.S., AND
CAPTAIN CHARLES B. TAYLOR, MEDICAL CORPS, A.U.S.

THE two-filter oximeter as designed by Millikan¹ is an instrument for measuring the per cent saturation of the blood hemoglobin with oxygen. The apparatus consists of an earpiece, a small control box, and a galvanometer. The earpiece is clamped on the ear and the apparatus is standardized while the subject breathes pure oxygen. After standardization, the per cent saturation of the blood hemoglobin with oxygen can be read from the galvanometer scale at any time. For ease and convenience, this method has considerable advantage over the chemical analytical method since it requires no arterial punctures and gasometric analyses. Furthermore, the value of the oxygen saturation can be obtained instantaneously, without a wait of twenty or thirty minutes for the results of a chemical analysis. At the present time this method has found its main use in the altitude indoctrination program, but its use can be extended to experimental human physiology and to operating room procedures.

The earpiece of the oximeter contains a small lamp placed on one side of the ear and two photoelectric cells on the other. Special Wratten filters are placed in front of each photocell. One filter (called the green filter) transmits light which is absorbed by both reduced and oxygenated hemoglobin, while the other filter (the red filter) transmits light which is absorbed by reduced hemoglobin. Light passing through the ear passes through the respective filters and is received by the photocell. The photoelectric current from the photocell with the green filter is inversely proportional to the total amount of hemoglobin plus oxyhemoglobin (ear thickness), while the photoelectric current of the red-filter photocell is inversely proportional to the amount of reduced hemoglobin in the light path. In the older instruments five galvanometer scales were used, the choice of scale depending on the reading of the green filter photocell. When vasoconstriction or vasodilation occurred in the ear, it was sometimes necessary to change from one scale to another during an experiment. In a more recent modification, an improvement has been made in which a single scale is used and the circuit has been changed to allow automatic adjustment for vasomotor changes. The details of the revision are given by Millikan.¹ Since the changes in the circuit involved some approximations which would reduce accuracy, it was necessary to perform a series of calibration experiments.

*On leave of absence from the University of Minnesota.
Received for publication, May 9, 1944.

METHOD

The oximeter circuits used are shown in Fig. 1. The photocells of the earpiece are designated by *G* (green) and *R* (red). A small panel box contains the potentiometers and the dpdt switch. When Coleman earpieces (Coleman Electric Company, Maywood, Ill.) were used, a Leeds and Northrup type R galvanometer 2500b (CDRX 10,000 ohms and sensitivity 0.0005 microamperes per millimeter at a scale distance of 1 meter) was found to have more than the required sensitivity and performed satisfactorily (see oximeter circuit *A*). Another oximeter circuit *B* was identical with circuit *A* except that in place of the high-sensitivity galvanometer, a direct-current amplifier was used with an Esterline-Angus recording milliammeter having a sensitivity of 1 milliamperes full scale. With circuit *B* a written record of the changes in oxygen saturation was obtainable, whereas with circuit *A* it was necessary to take galvanometer readings visually. A record from the recording oximeter is shown in Fig. 2 which illustrates the changes in per cent oxygen saturation of the blood when a subject breathing 100 per cent oxygen is changed to 8 per cent oxygen plus 92 per cent nitrogen and then returned to 100 per cent oxygen.

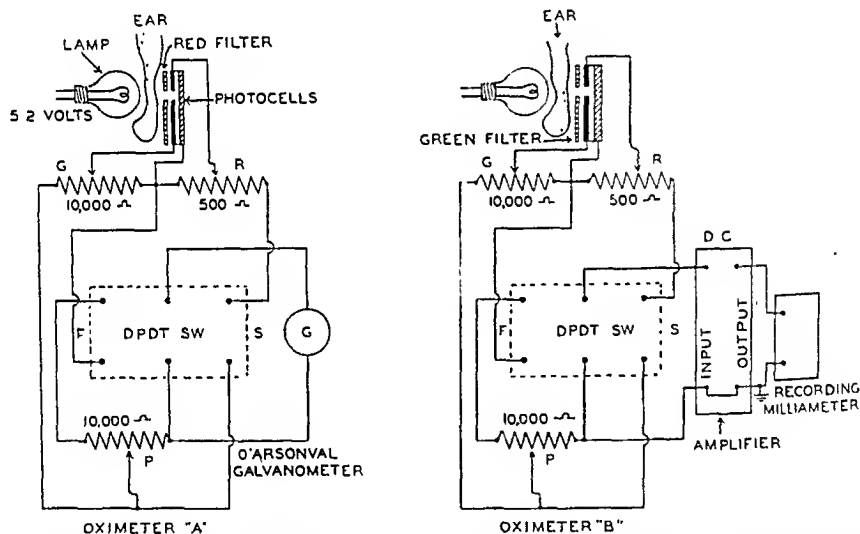


Fig. 1.—The two oximeter circuits used are identical except that the circuit labeled "galvanometer" has a d'Arsonval galvanometer to indicate blood oxygen saturation values and the circuit labeled "EA Recorder" uses a direct-current amplifier and an Esterline-Angus 1 millimeter recorder.

The electrical operation of this circuit is described in detail by Millikan.¹ The setting of the potentiometer *P* is determined by setting the galvanometer at zero in midscale and moving the sliding contact of the 500-ohm (red filter photocell) potentiometer *R* to zero. The dpdt switch is thrown to *S*, and *G* is adjusted until the galvanometer reads full scale. The switch is then thrown to the *F* position and *P* is adjusted until the galvanometer, now deflecting in the opposite direction, reads exactly $\frac{1}{2}$ of the reading in the *S* position. Once *P* is set it remains fixed for all readings.

The oximeter scale reads from 50 to 100 per cent on a logarithmic scale. In operation the galvanometer is adjusted to the scale zero (50 per cent on the

scale) and the "neutral filter" is placed in the earpiece. The apparatus is allowed to "warm up" for twenty minutes until thermal equilibrium is attained. The switch is thrown to *F* and *G* is adjusted until the galvanometer reads 100. The earpiece is then clamped on the subject's ear and allowed to remain for fifteen minutes until thermal equilibrium on the ear is attained. The subject then breathes 100 per cent oxygen, the switch is thrown to *S*, and potentiometer *R* is adjusted until the galvanometer again reads 100.

In testing the instruments, aviation students were used. They were fitted with oxygen masks and allowed to breathe pure oxygen while the preliminary standardization was being made. The breathing mixture was then changed to 8 per cent oxygen plus 92 per cent nitrogen. The oxygen content of the blood fell progressively as indicated by Fig. 2, and when the per cent saturation had reached 65 to 70 per cent, a sample of arterial blood was drawn under oil from the femoral artery. Oxygen content and oxygen capacity were determined by the Van Slyke manometric method and the per cent saturation computed. In a few experiments two samples of arterial blood were drawn, the first when the subject was breathing 100 per cent oxygen and the second when he was breathing 8 per cent oxygen.

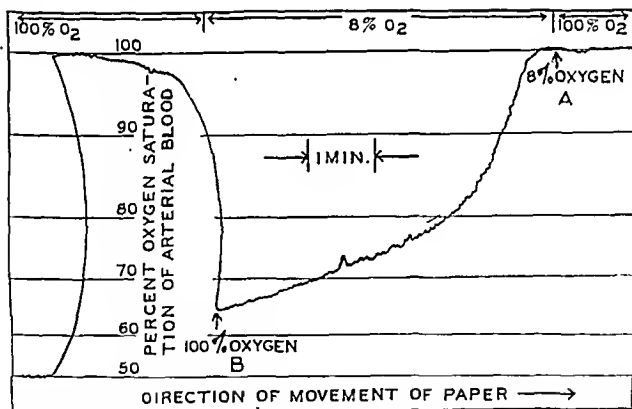


Fig. 2.—Record from recording oximeter. At the time indicated by *A*, the subject who was breathing 100 per cent oxygen was changed to 8 per cent oxygen plus 92 per cent nitrogen. At *B* the reverse change back to 100 per cent oxygen was made. In the interval *A-B*, the 8 per cent oxygen-92 per cent nitrogen was breathed.

RESULTS

The results are plotted graphically in Fig. 3. The electrical values read from the instruments are plotted on the abscissa, and the chemical values, obtained by computation from Van Slyke analyses, are plotted on the ordinates. Values obtained from oximeter *A* are plotted in Fig. 3 in the graph with "galvanometer" on the ordinate. Values for oximeter circuit *B* are in the graph with "EA recorder" in the ordinate. The line at 45° in the graph indicates

identical values. The two lines above and below indicate regions of ± 2 per cent and ± 5 per cent. It is to be noted that agreement within ± 5 per cent is obtained for 89 per cent of the values using oximeter *A* and 83 per cent of the values using oximeter *B*. In all, 37 experimental tests were performed.

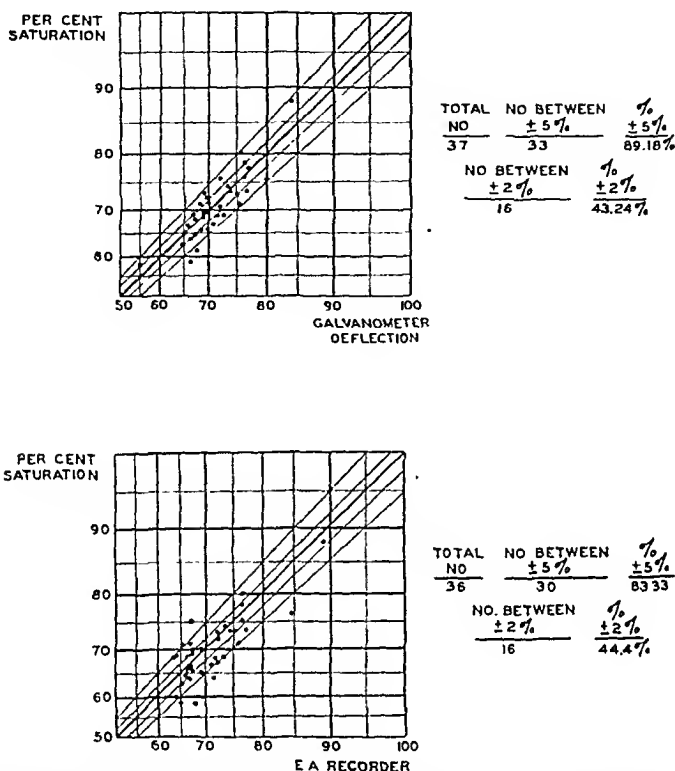


Fig. 3.—The galvanometer readings are plotted on the abscissa and the corresponding chemical oxygen analyses on the ordinate. For exact agreement, the points should fall on the heavy middle line sloping at 45° .

DISCUSSION

The agreement between the electrical and chemical values is approximately the same for both types of oximeter. It had been anticipated that the high-sensitivity galvanometer method (*A*) would give closer checks with chemical analyses than the amplifier method (*B*) due to the general unsteadiness with high amplification. The results show that this expectation was unwarranted and that the recording method gives values which agree well with the d'Arsonval galvanometer values.

It is believed that in this method of testing agreement of better than 5 per cent between electrical and chemical methods is not possible. There are certain random errors inherent in the method of testing which would account for a discrepancy of 2 to 5 per cent even if the oximeter gave the correct instantaneous values. These errors are the following:

a. *Errors in Chemical Analyses.*—The Van Slyke manometric method for blood oxygen is capable of giving duplicate values for oxygen content which agree within 0.5 per cent in the absolute value. The duplicates for oxygen

capacity are not as reliable but most of the values agree within 1.5 per cent. In terms of per cent saturation, the duplicates will have a maximum error of 2 per cent (in oxygen saturation) and usually check within 1 per cent. Also the Van Slyke method measures total blood oxygen, while the oximeter, recording the amount of reduced hemoglobin, measures only hemoglobin oxygen.

b. *Changing Oxygen Content of Blood.*—When subjects breathe 8 per cent oxygen plus 92 per cent nitrogen, there is a progressive fall in arterial oxygen content with the rate of fall varying from one individual to another. Superimposed on the more or less regular rate of fall of blood oxygen, there are fluctuations caused by deep and irregular breathing due to the carotid sinus stimulation. It requires thirty seconds to one minute to draw a sample of arterial blood, and in this time interval the arterial content varies in an amount equal to 1 to 5 per cent of the saturation value. The sample of blood in the syringe will represent an almost unpredictable average since it is drawn from the artery at rates which may vary.

c. *Error of Simultaneous Sampling*—This error, which is probably small and is estimated to be less than 1 per cent, is the error involved in simultaneity of oximeter readings and blood sampling. Since the blood oxygen content is changing during the period in which blood is withdrawn, an exact record of the oximeter readings in this period is necessary.

When these three sources of error are considered, it is possible to have a discrepancy between analytical and oximeter oxygen saturation values in an amount as high as 5 per cent if the oximeter recorded the true oxygen value. These errors are inherent in the method of testing and are not errors due to faulty performance of the oximeter. The over-all average values differ by less than 1 per cent. For these reasons the error involved in using the oximeter is believed to be well within the value of 5 per cent which can be considered as a maximum.

SUMMARY

The Millikan type oximeter with automatic compensation for ear thickness has been tested by chemical analysis of arterial blood drawn from subjects breathing a mixture of 8 per cent oxygen and 92 per cent nitrogen. Agreement within ± 5 per cent was obtained for values of oxygen saturation as low as 60 per cent. Two instruments were tested and found to give similar values. One instrument used a d'Arsonval galvanometer and the other a recording milliammeter with a direct current amplifier.

REFERENCE

1. Millikan, G. A.: The Oximeter, an Instrument for Measuring Continuously the Oxygen Saturation of Arterial Blood in Man, *Rev. Scient. Instruments* 13: 434 1942.

MODIFIED KOCH PIPETTE FOR CARR-PRICE REAGENT DISPENSER

D. B. PARRISH, M.S., AND M. J. CALDWELL, M.S., MANHATTAN, KAN.

IN THE Carr-Price method of determining the Vitamin A content of blood¹ and other biological materials, difficulty is often experienced due to the reaction of the reagent with atmospheric moisture. If the antimony trichloride reagent is allowed to come in contact with the water vapor of the air, the inner surface of the delivery pipette soon becomes encrusted with the reaction product; hence special precautions must be observed in the use of the reagent.

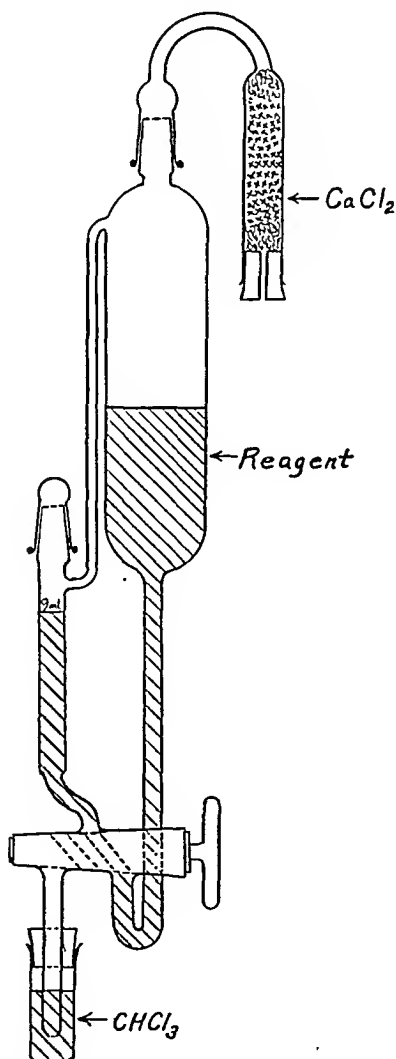


FIG. 1.—Modified Koch pipette for Carr-Price reagent dispenser.

From the Kansas Agricultural Experiment Station. Contribution No. 290, Department of Chemistry.

Received for publication, July 3, 1944.

Recent publications of Oser et al.² and Swain³ have described special apparatus for dispensing the reagent. The difficulties encountered in handling the Carr-Price reagent have been largely eliminated in our laboratory by the use of the modified Koch pipette shown in Fig. 1.

The pipette is built around the three-way, large-bore (3 mm.) stopcock which will deliver 9 c.c. of reagent in approximately two seconds. The reservoir of about 200 c.c. capacity is connected to the under side of the stopcock by means of a 180° bent tube, which also serves as a settling chamber. The air breathing into and out of the pipette has been predried by passage through a calcium chloride tube and is in equilibrium with the antimony trichloride solution in the reservoir. The pipette will remain free of hydrolyzed products indefinitely. Fresh solutions of the reagent are introduced by funnel into the top of the reservoir, which is closed by means of a ground glass fitting, connected to a calcium chloride tube.

In use, the pipette is supported on a ring stand directly above the reaction cell in the photometer. The rapid delivery of 9.0 c.c. of reagent to 1.0 c.c. of the chloroform solution of vitamin A insures adequate mixing. After delivery of the reagent, a filter paper is touched to the delivery tip to remove the residual drop of solution. The tip is kept submerged in chloroform when the apparatus is not in use, and remains clean.

Since all parts of the apparatus which come in contact with the antimony trichloride solution are of glass, there is little chance of contamination. A good grade of stopcock grease used in moderation prevents leakage and has not been observed to interfere with the determination. The fact that the reagent may be allowed to remain in the apparatus from day to day results in considerable convenience and saving of time.

REFERENCES

1. Kimble, M. S.: The Photocolorimetric Determination of Vitamin A and Carotene in Human Plasma, *J. LAB. & CLIN. MED.* 24: 1055, 1939.
2. Oser, B. L., Melnick, D., and Pader, M.: Estimation of Vitamin A in Food Products, *Ind. Eng. Chem., Anal. Ed.* 15: 724, 1943.
3. Swain, Lyle A.: A Carr-Price Reagent Dispenser, *Ind. Eng. Chem., Anal. Ed.* 16: 241, 1944.

length of the specimen. The sides must be low, so that the light reflected from the top and bottom edges of the sides and refracted through the solution will not form streaks in the field of the picture.

BACKGROUND

We have described our use of colored backgrounds in detail in another publication.¹ We are convinced that their use not only gives pleasing results but also aids in the visualization of the structures intended to be portrayed; but in no situation are the results more striking than in the photography of small specimens. Briefly, we use an illuminated box with the lights at the bottom; the specimen, in this case the tank containing the specimen, is placed on a plate glass above. About 6 inches below the upper glass plate a colored glass is inserted. The color of the background varies not only with the color of the glass and the strength of the lights beneath it, but with the intensity of the incident light on the specimen.¹

PHOTOGRAPHIC EQUIPMENT

Any equipment which takes satisfactory color pictures with fresh specimens can be used in this method. A large aperture (f:4.5) lens is necessary for focusing when a scale of $\times 1$ or $\times 2$ is desired. Proper lamps, 3200° Kelvin, 500 W, in reflectors for Kodachrome type B film are indispensable for good color rendering. We use $3\frac{1}{4}$ by $4\frac{1}{4}$ inch film because we think the better results more than justify the additional expense over miniature film.

EXPOSURE

When the specimen is twice the diameter of the photo-electric cell of the usual exposure meter or larger, there is no difference between obtaining the proper exposure in this from ordinary color photography. If the specimen is small, the exposure can be obtained with reasonable accuracy by placing the palm under the plate glass beneath the specimen. In any case, while measuring exposure the lights of the illuminated box *must be turned off*.

A few specimens with which we have worked require slight modifications from the method mentioned.

EYES

Rarely does one desire to photograph the outside of an enucleated eye. External pathology is better photographed on the patient before the eye is removed. Cross sections are exceptionally difficult to make without dislocating the lens and otherwise disturbing anatomic relations; good sections are practically impossible on a fresh eye. The following method usually gives good results, but even with the utmost care one will sometimes displace or entirely dislodge the lens.

The eye is fixed in formalin for 24 to 48 hours and washed in water. Over a period of 24 hours it is then put in ascending strengths of alcohol, starting in 50 per cent and ending with 95 per cent. Then a single cut section is made, slightly above the level of the outer edge of the iris. The specimen is then carefully lowered into alcohol for several hours of additional hardening. The last section, parallel to the first, is then made.

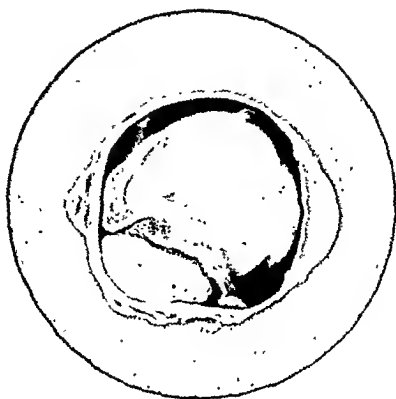


Fig. 1.—Leucoblastoma of the eye. Detachment of the retina. Fixed in formalin 4 days, then sectioned. Photographed in tank under alcohol.



Fig. 2.—Achondroplasia. Transected femur and tibia. Fixed in formalin 24 hours. Photographed in tank under alcohol.

BRAINS

Many pathologists prefer to fix brains immediately upon removal. Certainly a fresh brain is not easy to handle and, if cut into, anatomic details may be obscured. Good results may be obtained if the brain is cut in four or five days, and the sections for photography are placed in alcohol. Since we have no tank large enough for a complete brain section, we either wash the specimen in water after it is removed from the alcohol and photograph it as we would a fresh specimen, or we keep it moist with alcohol, by the use of a dropping bottle, while it is on the plate of our box. The latter method is often to be preferred because some specimens become grayish after washing in water.

The successful photography in color of brains after fixation is probably the result of the small range of color in this organ. However, the delicate colors of meningeal purulent exudates are not too well preserved so that if possible such brains should be photographed while fresh.

OTHER ORGANS

External views of kidneys are fairly good after fixation. Sometimes the details of speckled lesions, such as in embolic nephritis, show up even better than in the fresh state, although the color of the renal tissue itself may not be exactly as it was when fresh. Sections of the spleen may be difficult to photograph fresh especially if, as in leucemia, they develop a film of coagulation on exposure to air. In such cases a section about 1 cm. thick may be fixed for 24 hours, a thin slice cut off the top and then treated as above. Fixed hearts give mediocre results. We have never obtained even a passably good picture of a fixed lung.

REFERENCE

1. Beiter, J. J., and Bohrod, M. G.: Transilluminated Color Backgrounds in Medical Photography, *J. Biol. Phot. A.* In press.

BOOK REVIEWS AND NOTICES

Hypertension and Hypertensive Disease. By William Goldring, M.D., Associate Professor of Medicine, New York University College of Medicine; Chief, Nephritis and Hypertension Clinic, New York University Clinic; Physician to the Adult Cardiac Clinic and Associate Visiting Physician, Third (New York University) Medical Division, Bellevue Hospital; Visiting Physician, Goldwater Memorial Hospital, and Herbert Chasis, M.D., Assistant Professor of Medicine, New York University College of Medicine; Associate Chief, Nephritis and Hypertension Clinic, New York University Clinic; Physician to the Adult Cardiac Clinic and Assistant Visiting Physician, Third (New York University) Medical Division, Bellevue Hospital; Cardiologist to the French Hospital. The Commonwealth Fund, 41 East 57th Street, New York 22, New York. Price \$3.50. Cloth with 253 pages.

The purpose of this book is a "concise presentation of our (the authors') concepts of hypertension and hypertensive disease." It is recognized that "such a book must contain some dogmatism." The discussions of the possible identity of essential and experimental renal hypertension, of the uses of thiocyanate and nephrectomy, of the origin of hypertension in renal ischemia—to all of which they are opposed—vigorously sustain this purpose. The interest of these discussions is dulled by the insertion of beautifully printed tables—for instance, Tables 17 and 18—which abundantly and uselessly confirm points clearly stated in the text. The sections devoted to treatment are not completed by specific recommendations which might be of value to those less expert than the authors.

Throughout, the text is well written, and wherever it has held fast to the authors' intention, the presentation is excellent. When, to complete the argument, they find it desirable to stray from this purpose, concepts are introduced which, because of brevity, are necessarily incomplete, and therefore, sometimes misleading. The reader must carefully disentangle the serious opinions of the authors from the opinions they here attribute to others. The appendix contains descriptions of the measures they use for determination of cardiac output and renal function, and a discussion of the interpretation of renal functional data which should interest investigators in this field.

The student or clinician who would like to know the special point of view held by the authors will find it clearly presented in this book. Their experience and contributions have made that opinion well worth knowing, and worthy also of the dignified format selected by the publishers. The promise of a more rounded survey, implied in the title and suggested by the jacket blurb is not fulfilled. This, however, was not the authors' intention.

IRVINE H. PAGE, M.D.

Heart Diseases. By Paul Dudley White, M.D., Lecturer in Medicine, Harvard Medical School; Physician to the Massachusetts General Hospital, Boston. Third Edition, The Macmillan Company, 60 Fifth Avenue, New York. Price \$9.00. Cloth with 1025 pages.

The new edition of this book maintains the high standards of the two previous editions. It is a skillful, comprehensive survey of diseases of the heart. Rapid advances in cardiology since the publication of the second edition in 1937 have necessitated a thorough revision. Significant alteration and augmentation have been made so that the book is larger than previously. A notable addition is the new first chapter on "The Range of the Normal Heart." Other important added sections include those on phonocardiography, recent knowledge concerning splanchnic resection for hypertension, a discussion of the surgical therapy of the patent ductus arteriosus, newer aspects of the treatment of subacute bacterial endocarditis, and an adequate and clear presentation of the present knowledge of precordial leads. Other new sections include intracardiac thromboses, pulmonary embolism, circulation of the blood and the cardiac aspects of military service, gastrointestinal diseases and miscellaneous conditions.

The book contains more illustrations than the previous edition. These are very well arranged. One of the most valuable features is the extensive and carefully chosen bibliography which follows every chapter. For a textbook covering a subject so full of details as cardiology, very little of importance has been omitted, although obsolete and unimportant material has been excluded.

A very worth-while chapter is the Addendum in which the author briefly presents information of current importance accumulated since the main body of the manuscript was sent to press. The volume is admirable for the clarity with which it is written and is highly recommended.

EDWARD MASSIE, M.D.

The Analysis and Interpretation of Symptoms. By Cyril M. MacBryde, Assistant Professor of Clinical Medicine, Washington University School of Medicine; Director, Metabolism Division, Barnes Hospital, St. Louis, Missouri, J. B. Lippincott Company, Philadelphia. Cloth with 301 pages.

In textbooks of medicine it is customary to present pictures of disease with combinations of symptoms which, taken together, enable the reader to recognize the conditions at the bedside. Mere recognition, however, does not necessarily imply understanding. It may be only through the analysis of each component manifestation that disease can be truly understood. Moreover, in the field of therapy mere recognition of a clinical entity is practically sufficient only when a specific remedy is available. In the absence of a specific remedy, treatment must depend upon the relief of individual symptoms, deep understanding of which is essential to effective management. For these reasons alone *Analysis and Interpretation of Symptoms* reprinted in book forms from Clinics, 1944, constitutes an important contribution. In the introduction the editor outlines admirably the purposes of the volume. He emphasizes that the analysis and interpretation of a symptom must depend primarily upon understanding of functional pathology, while its correlations with other clinical and labora-

tory manifestations and its relations to other diseases, although important, must be given secondary consideration. Nine subjects are discussed. It is appropriate that four of them relate to pain, the most common and troublesome of all clinical phenomena. The selection, as examples, of headache and of thoracic, abdominal, and joint pain permits wide scope in discussion. There is an excellent chapter on fever and a challenging discussion of nervousness and fatigue. Cough and hemoptysis are considered in their relation to various parts of the respiratory tract. Hematemesis and melena are competently considered both from the standpoint of their pathogenesis and their clinical relationships. There is a most stimulating chapter on obesity and a short but comprehensive discussion of jaundice.

Although in general the selection of topics has been fortunate, it is obvious that only a very few of the symptoms which are appropriate for similar treatment have been considered. It is to be hoped that these valuable and interesting discussions may be used as a nucleus for a more comprehensive book on the analysis and interpretation of symptoms.

DAVID P. BARR, M.D.

The Journal of Laboratory and Clinical Medicine

Vol. 29

OCTOBER, 1944

No. 10

PROGRESS

THE PROGNOSTIC SIGNIFICANCE OF AN ELEVATED BLOOD CREATININE

VICTOR C. MYERS, PH.D., D.Sc., CLEVELAND, OHIO

IT IS now thirty years since it was first observed that the creatinine of the blood is markedly elevated in cases showing severe impairment of renal function. Since the initial observations were made a number of controversies have arisen involving not only the validity of the determination itself but also the interpretation which can justly be placed upon the results obtained. It is believed that we now possess sufficient facts and a sufficiently long perspective to adequately evaluate the accuracy and clinical importance of this determination.

Before entering upon a discussion of the creatinine of the blood it may be well to indicate some of the most fundamental facts regarding creatine-creatinine metabolism. Folin¹ was the first to show that the amount of creatinine excreted in the urine by a normal individual on a meat-free diet is quite independent of either the amount of protein in the food or the total nitrogen in the urine, the amount excreted from day to day being practically constant, thus pointing conclusively to its endogenous origin. Creatinine is the anhydride of creatine and one might assume on a *priori* grounds that creatine is the precursor of creatinine. However, this was questioned by no less an authority than Folin himself. The origin of creatine was even more obscure, although logic and some experimental data suggested that arginine might be the precursor. Very recently Borsook,² du Vigneaud,³ Schoenheimer,⁴ and their coworkers have definitely answered the question regarding the origin of creatine, and have conclusively proved that creatinine originates from creatine. du Vigneaud and Schoenheimer were able to arrive at their conclusions by the use of tracer isotopes. The im-

From the Department of Biochemistry, School of Medicine, Western Reserve Univ.
Received for publication, July 20, 1944.

mediate precursor of creatine is glycoeyamine. This is formed from glycine and arginine (also citrulline) in the kidney, while glycoeyamine is methylated in the liver by methionine (or a derivative of methionine) to form creatine.

The importance and significance of muscle creatine was not appreciated until Fiske and Subbarow⁵ in Folin's laboratory in 1927 showed that in living muscle creatine is present very largely in combination with phosphoric acid, probably as a potassium salt. Quite as Folin and Denis⁶ prophesied in 1914, creatine has been found to be a post-mortem product. In the process of fatigue and death phosphocreatine (or creatine phosphate) breaks up into its constituent parts. Although the complete story of the energetics of muscular activity has not been told, it is evident that creatine phosphate and other compounds of phosphoric acid are vitally concerned in the production and transfer of energy. Creatine is a necessary link in this chain. In man the normal creatine concentration of voluntary muscle is about 440 mg. per 100 grams of muscle, less than half this concentration (about 200 mg.) in the left ventricle of the heart, still less in the brain (about 170 mg. in the cerebellum and 110 mg. in the cerebrum), and much less in smooth muscle (about 60 mg.).

CREATININE CONTENT OF THE BLOOD

Folin and Denis⁷ were the first to present any very extensive data on the creatinine content of the blood, although almost simultaneously Neubauer⁸ reported an observation on one case of "uremia," while Myers and Fine⁹ presented several analyses on two cases of nephritis showing marked retention of creatinine. Folin and Denis gave observations on nine cases of "uremia" and were the only observers to give figures for normal human blood. Shaffer¹⁰ had begun a study of this question some years previously and had made a preliminary report with Reinoso,¹¹ but unfortunately did not give his protocols until after the appearance of the paper by Folin and Denis.

Normal Creatinine Content of Blood.—Nearly all the original analyses were made on whole blood after precipitating the proteins with picric acid. The Jaffé color reaction was carried out directly on the blood filtrate by the simple expedient of adding 0.5 c.c. of 10 per cent sodium hydroxide to 10 c.c. of the saturated picric acid filtrate. With this procedure creatinine values between 1 and 2 mg. per 100 c.c. are obtained for normal individuals, being nearer 1 than 2 mg. As soon as one passes to hospital patients, however, slightly higher values may be encountered, especially if whole blood is employed for analysis. Although the great majority of cases without renal involvement show creatinine figures on the whole blood below 2.5 mg. per 100 c.c., very occasionally figures as high as 3.5 mg. may be encountered that are not readily explained.

Although most of the earlier analyses were made on whole blood by the Folin method¹² or the Myers and Fine modification,¹³ it was later shown by Hunter and Campbell¹⁴ that there is present in the corpuscles a substance which interfered, and tended to raise materially the values obtained on whole blood. Obviously the percentage of error here would be greatest in normal blood with a low concentration of creatinine. On this account these authors recommended the use of plasma or serum. Later methods employing other protein precipitants and a lower concentration of picric acid, thus making the Jaffé color reaction more specific for creatinine, tended to lower the values obtained on normal blood.

Consequently it would appear that strictly normal human plasma contains between 0.5 and 1.5 mg. creatinine per 100 c.c. with an average of close to 1.0 mg.* Folin and Svedberg¹⁷ obtained somewhat similar figures on Folin's "unlaked blood" filtrate.

Before discussing the clinical significance of abnormal amounts of creatinine in blood it may be well to discuss briefly the question that has been raised as to whether or not creatinine is actually present in blood.

Controversy on the Presence of Creatinine in Blood.—In 1922 Behre and Benedict¹⁶ presented evidence to show that creatinine does not exist in normal blood. The facts upon which they made this deduction were: (1) that creatinine can be removed from blood by bone black, but bone black does not affect the chromogenic substance present in blood filtrates, which reacts with picric acid and sodium hydroxide; (2) that a substance present in picric acid blood filtrates gives a good reaction with sodium carbonate, and this reaction is increased in bloods showing an abnormally high "creatinine" content, whereas pure creatinine in picric acid is only slightly affected by carbonates; (3) that creatinine is readily destroyed by heating in alkaline solution, but the chromogenic substance present in blood is not appreciably affected; (4) that kaolin completely removes small amounts of creatinine from blood, but does not affect the picric acid-alkali reacting substance. Their observations were confirmed, and two additional observations support this view. (1) Benedict and Behre¹⁷ found that the color obtained on blood filtrates and dilute serum ultrafiltrates with 3, 5-dinitrobenzoate differs from that of creatinine, and (2) the apparent creatinine of normal dog serum ultrafiltrates (Gaebler¹⁸) and plasma filtrates of human and other species (Behre and Benedict¹⁹) fail to precipitate under conditions which precipitate added creatinine. Shortly after the appearance of Behre and Benedict's original paper, Folin²⁰ commented: "In view of the many still active investigators who in the past have made contributions to the creatine-creatinine problem one can safely predict that the findings and conclusions of Behre and Benedict will not remain without contradiction or verification."

Quite as Folin prophesied the observations and interpretations of Behre and Benedict led to a bitter controversy. There is no question about the presence of creatinine in urine. It would appear therefore that the kidney must (1) either separate and concentrate creatinine present in blood, or (2) form creatinine from a closely related precursor compound present in the blood, and then excrete it. If the observations of Behre and Benedict are correct, the latter would serve to explain the discrepancies. The opponents of Behre and Benedict, however, feel that this explanation is unnecessary. Hayman, Johnston and Bender²¹ found that when trichloroacetic acid (rather than picric acid) was employed as the protein precipitant on serum or plasma, the filtrate differed little in its reaction from pure creatinine. Danielson²² came to much the same

*Arkin, Popper and Goldberg²³ have recently recommended the determination of plasma creatinine as a test of low grade kidney damage. They consider the normal range to lie between 0.60 and 0.95 mg. per 100 c.c., and regard any increase over 1.0 mg. as a probable sign of impaired glomerular filtration. They feel that the test in the low ranges is superior to the determination of the nonprotein nitrogen and urea, not only for theoretical reasons (lack of reabsorption in the tubules) but also because of the sharper limits between the normal and pathological range, and consequently provides a helpful diagnostic procedure for the detection of low grade kidney damage. The test is regarded as of diagnostic and prognostic importance in acute nephritis, chronic nephritis, nephrosis, nephrosclerosis, urologic conditions and heart failure.

conclusion from a study of plasma ultrafiltrates. With the aid of a specific bacterial enzyme, Miller and Dubos²³ have approached the problem in a different way and conclude that in normal individuals creatinine constitutes 80 to 100 per cent of the chromogenic material in serum or plasma.

Gaebler in particular has made important contributions to a solution of this problem. With Kelteh²⁴ and independently²⁵ he demonstrated that creatinine can be isolated from normal blood, and in quantity from retention blood. The creatinine thus isolated was definitely identified chemically. Although originally opposing Behre and Benedict, he²⁵ later agreed that creatinine as such was not present in detectable amounts in normal blood. More recently Gaebler²⁶ and Gaebler and Abbott²⁷ have shown that most of the apparent creatinine of ultrafiltrates of normal blood can be precipitated with picric acid and rubidium. Goudsmit²⁸ has compared the apparent creatinine content of renal venous blood and of arterial blood, and found the former consistently lower, thus definitely indicating that the apparent creatinine of the blood is the precursor substance of urinary creatinine.

It is evident therefore that the apparent creatinine of blood, whether creatinine itself or a precursor, can be converted in the test tube, in considerable part at least, to creatinine and can be so converted by the kidney. Consequently the apparent creatinine of blood, if not creatinine, has the same significance in blood as creatinine.

CREATININE CONTENT OF THE BLOOD IN DISEASE

In general it may be stated that the concentration of creatinine in the blood (plasma) is not significantly altered in disease, except where marked impairment in renal function has occurred. While normal renal function is primarily dependent upon normal kidneys, it is also dependent upon normal blood flow to the kidneys. This may be sufficiently impaired in marked dehydration and severe heart disease to have a definite influence on the level of the nitrogenous waste products in the blood, including creatinine. Practically it has been found that there is no indication for making a blood creatinine determination until after the level of the blood urea has exceeded the upper normal limits.

It should be noted that the older methods on whole blood have occasionally yielded moderately elevated creatinine values (figures between 3 and 4 mg. per 100 c.c.) in syphilis, in certain heart conditions, sometimes in fevers, such as pneumonia, in advanced malignancies, and in some cases of severe diabetes. It is well known that an increased formation of creatinine occurs in fevers, while in diabetes the influence of the high glucose concentration on the Jaffé color reaction for creatinine accounts in part for the elevated values in some cases.

High values for the creatinine of the blood (over 4 mg. per 100 c.c.) have been observed in a variety of conditions in which renal function is seriously impaired. The following conditions may be mentioned: various forms of Bright's disease, but chiefly glomerulonephritis and nephrosclerosis, pyelonephritis, bichloride poisoning, double polycystic kidney, prostatic hypertrophy, vesical neoplasms, intestinal obstruction, acute suppression of urine from various causes, etc.

Prognostic Value of the Blood Creatinine - Attention was apparently first called to the unfavorable prognostic import of an elevated blood creatinine by Myers and Lough²⁹ in 1915. They suggested that since creatinine was almost wholly endogenous in origin and, furthermore, since it was apparently the most readily eliminated of the three nitrogenous waste products, uric acid, urea and creatinine, it should be of greater prognostic value than either uric acid or urea. A year later Chace and Myers³⁰ further discussed this subject and stated on the basis of the generally accepted average figures for the concentration of these three constituents in the blood and urine, that whereas the kidney normally concentrates the uric acid about 20 times, and the urea about 80 times, it concentrates the creatinine about 100 times. More recent studies by Mayrs,³¹ Rehberg,³² MacKay and Cockrill,³ and Cope,³³ dealing with the concentration ratios of administered creatinine and urea, definitely showed that the kidney is able to concentrate creatinine to a greater extent than urea, and thus bear out the original contention of Myers and his coworkers. In this connection some observations of Berglund and Frisk⁴ are also of interest. They have compared the ability of the kidney to excrete creatinine with uric acid. They state: "Whether measured directly or under the influence of drugs . . . the efficacy of uric acid elimination is greatly inferior to the elimination of creatinine. Uninfluenced by drugs the elimination is a fifth to an eighth only as efficacious as the mechanism for creatinine elimination."

Myers and Lough²⁹ stated in their original paper: "The creatinine rises above 2.5 mg. per 100 c.c. of blood almost without exception only in conditions with renal involvement. Creatinine values from 2.5 to 3.0 mg. may be viewed with suspicion; figures from 3.0 to 5.0 mg. regard as decidedly unfavorable, while over 5.0 mg. probably indicates an early fatal termination." Although with improvements in methods the values obtained for the creatinine of the blood have tended to fall slightly, the above statement, made nearly thirty years ago, would still appear to stand.

In 1919 Myers and Killian³⁴ again discussed the prognostic value of the creatinine of the blood in nephritis on the basis of 100 cases with over 4 mg., and 85 cases with over 5 mg. creatinine per 100 c.c. of blood (5.33 mg.), studied between March, 1914 and January, 1919. Of the 85 cases, 80 were dead at the time of their publication, 3 died shortly afterwards and 2 recovered. The 2 cases which recovered showed only a temporary elevation of the creatinine above the 5 mg. level. In this connection Myers and Killian commented: "In such conditions as bichloride poisoning and acute nephritis it is possible to have considerable nitrogen retention with ultimate recovery, for the reason that here the activity of the kidney is only temporarily depressed." The authors noted that a considerable number of the 85 cases were able to be up and about and some showed decided clinical improvement. They felt that the following of the blood creatinine gave them a better prognostic insight into these cases than either the blood urea or the phenolsulfonphthalein tests which were done simultaneously.

Case E. M. is a good illustration of a patient in the last stages of the disease, but who, nevertheless, was able to be up and about (see Table I).

Case 2. J. B. illustrates the prognostic value of the blood creatinine particularly well. This patient's blood showed a creatinine of 7.5 mg. when he first

TABLE I
SERIAL OBSERVATIONS ON FOUR CASES ILLUSTRATING THE PROGNOSTIC VALUE
OF THE BLOOD CREATININE

CASE	DATE	CHEMICAL BLOOD ANALYSES			
		CREATININE	UREA N	URIC ACID	CO ₂ CAPACITY
		mg.	mg.	mg.	c.c.
1. E. M.*	1915				
	Nov. 30	17.5	97	6.6	24
	Dec. 4	21.5	129	6.4	21
	Dec. 10	22.3	132	7.0	26
	Dec. 17	24.2	150	5.6	15
	Dec. 21	28.6	186	5.0	18
	Dec. 24	26.7	200	7.7	12
2. J. B.†	1916				
	Dec. 22	7.5	60		32
	1917				
	Jan. 5	9.7	135		
	Jan. 9	12.5	110		49
	Jan. 16	9.5	93		
	Jan. 30	7.8	48	5.00	
	Feb. 13	7.8	51	6.4	51
	Feb. 27	7.5	45	7.1	
	Mar. 6	6.4	49	6.1	
	Mar. 20	10.6	53		
	Apr. 3	7.2	49		
	Apr. 13	6.4	46	5.9	
	Apr. 27	7.5	45		
	May 15	7.3	33		
	May 22	7.3	24		
	June 5	6.7	34		
	July 17	6.8	29		
3. C. W.‡	1921				
	Jan. 17	11.0	90		
	Jan. 26	11.1	111		
	Feb. 16	6.0	40		37
	Feb. 23	6.8	32		37
	Mar. 2	5.3	31		
	Mar. 10	4.9	35		28
	Mar. 18	4.6	37		44
	Apr. 13	7.1	49	4.8	31
	Nov. 4	5.1	41	6.5	35
	1922				
	Jan. 31	4.2	44	10.1	37
	Feb. 11	4.1	38	5.2	38
	1923				
	Sept. 4	9.2	60	5.3	
4. J. C.§	1917				
	Apr. 24	3.8	28	6.8	50
	May 8	6.1	41	5.5	
	May 15	5.2	30	7.7	
	May 22	3.3	28	7.7	
	June 1	3.1	36	10.4	
	June 8	4.5	26		
	1918				
	Apr. 2	3.8	57		
	Apr. 26	4.5	51	6.7	
	1919				
	Jan. 19	4.3	64		34
	1920				
	Feb. 18	7.2	56	5.0	

*1. E. M., male, aged 39 was up and about on Nov. 30, did not realize the seriousness of his condition, had stopped in New York on his way to Florida for the winter; systolic blood pressure 240 mm. Hg; 'phthalein output 0 on three examinations; sp. gr. of urine continuously 1.005 on Dec. 10; died on Dec. 25.

†2. J. B., male, aged 34 kept on low protein diet for five months; discharged from hospital clinically improved on June 9; returned to work as guard on subway; died Nov. 7, 1917.

‡3. Col. C. W., male, aged 55, enlarged prostate and chronic nephritis, *cystotomy* Jan. 19, and *prostatectomy* Mar. 25, 1921; 'phthalein output Feb. 11, 1922 5 per cent; died Oct. 11, 1923.

§4. J. C., male, aged 60 continued active during most of this period as club manager, although he spent a number of rest periods in hospital beginning in April 1916; 'phthalein output 7 per cent on May 22, 1917; died in June 1920.

came under observation, and although he showed considerable clinical improvement over a period of nearly seven months, still his blood creatinine remained constant, except for a temporary elevation to 12.5 mg. The dietetic measures which were employed to alleviate the nitrogen retention resulted in a gradual reduction in the urea nitrogen from 135 mg. per 100 cc. to a low of 24 mg. Despite his high creatinine (last determination 6.8 mg.), the patient left the hospital feeling quite well, returned to work as a guard on the subway and did not die until about five months later.

The prognostic value of the creatinine is likewise well illustrated by the data on *Case 3 C. W.* (Table I), a case of chronic nephritis complicated by prostatic hypertrophy. On admission the creatinine was 11.0 mg., but 13 months later after a prostatectomy had dropped to a low of 4.1 mg. Six weeks after cystotomy the urea N dropped from a high of 111 to a low of 31 mg. The temporary increased elevation of both the blood creatinine and urea N following both surgical operations is of interest. Although this patient remained quite active for about two years following his prostatectomy, the ultimate outcome was clearly indicated by the blood creatinine, and he died about 2 years and 9 months after first admission to the hospital.

Since cases that recover, or live for some months, after the creatinine of the blood has exceeded 5 mg. per 100 cc. are exceptional, such cases merit special consideration. The individual observations on *Case 1 J. C.* (Table I) are of interest in this connection. This case remained nearly stationary for a longer period than any other in the series. On his last admission to the hospital the patient was definitely worse and the creatinine had risen to 7.2 mg. He died about four months after leaving the hospital. Even among cases having very high blood creatinine there were many who were able to be up and about and some who showed considerable clinical improvement. It was in these cases that the blood creatinine gave a particularly good prognostic insight into the true nature of the condition.

A study of 1600 (about 1300 additional) creatinine determinations on cases with values above 4 mg., up to January, 1929, did not lead Myers and Killian to alter the view expressed 10 years earlier.

In the author's book on the chemistry of the blood³⁷ a table is presented giving observations on seven cases of intestinal obstruction made by Killian in 1921. It is of interest to note that, of the seven cases the five who died, all had blood creatinine above 5 mg. per 100 cc., the range being from 5.2 to 17.0 mg. The two cases which recovered showed maximum creatinine values of 2.2 and 4.7 mg. respectively.

Comparing the creatinine and urea, Myers and Killian³⁸ state: "Theoretically, the amount of the increase of the creatinine of the blood should be a safer index of the decrease in the permeability of the kidney than the urea, for the reason that creatinine on a meat-free diet is entirely endogenous in origin and its formation (and elimination normally) very constant. Urea on the other hand, is largely exogenous under normal conditions and its formation consequently subject to greater fluctuation. For this reason it must be evident that a lowered nitrogen intake may reduce the work of the kidney in eliminating urea, but cannot affect the creatinine to any extent. Apparently the kidney

is never able to overcome the handicap of a high creatinine accumulation. It would seem that creatinine, being almost exclusively of endogenous origin, furnishes a most satisfactory criterion as to the deficiency in the excretory power of the kidneys, and a most reliable means of following the terminal course of the disease, though it should be noted that urea, being largely of exogenous origin, is more readily influenced by dietary changes, and therefore constitutes a more sensitive index of the response to treatment."

Another factor which may play a role in the greater prognostic significance of an elevation of the blood creatinine than the blood urea is the difference in the mechanism by which these two constituents are eliminated by the kidney.³⁸ Urea is apparently eliminated entirely by glomerular filtration and tubular reabsorption of water, although there is appreciable back diffusion owing to the great diffusibility of urea. While the chief factor concerned in the excretion of creatinine is again glomerular filtration and tubular reabsorption of water, there is not only no back diffusion but also the tubules of the human kidney have the ability to add (secrete) an appreciable amount of creatinine to the glomerular filtrate.

The reliability of elevated blood creatinine values as an unfavorable prognostic sign in terminal renal insufficiency was quickly and amply confirmed by workers in various parts of the world. Among these may be mentioned Rosenberg³⁹ Feigl,⁴⁰ Rabinowitch,⁴¹ Moreau and Diamant,⁴² Jeanbrau and Cristol,⁴³ Feinblatt,⁴⁴ Hubbard,⁴⁵ Cantarow and Davis,⁴⁶ Warter,⁴⁷ and Popper, Mandel and Mayer.⁴⁸ Although most workers have supported fully the conclusions of Myers and Lough,²⁹ a few have felt that the creatinine gives little or no more information than the blood urea. Peters and Van Slyke,⁴⁹ in particular have emphasized this point although admitting that the "gravity of a high blood creatinine above 5 mg. per 100 c.c., a nonprotein nitrogen above 100 mg., or a urea nitrogen above 80 mg. in the blood is of serious import. Usually creatinine and urea rise together but when they do not the creatinine may be either the first or last to indicate the approach of uremia." Hubbard⁴⁵ likewise observed that the "urea and creatinine concentrations in the blood parallel each other." He felt that sudden changes in blood urea concentration were probably due more to variations in the rate of urea formation, than to differences in the permeability of the kidney for these two compounds.

However, this has not been the general conclusion of most workers in this field. Patch and Rabinowitch⁵⁰ state in this connection: "A large series of observations led to the finding of cases in which the general relationships between the urea and creatinine concentrations of blood were absent. In these cases, high values were found associated with normal or nearly normal values. . . . It was found that, in spite of high urea values, symptoms of uremia were usually absent when the creatinine values were normal or nearly normal. It was also found that though high creatinine values and symptoms of uremia were accompanied by positive diazo color reactions, this was not necessarily so with high urea values. . . . The clinical value of these observations, from the point of view of the chemical reactions of the blood, lies in their showing that urea studies unaccompanied by observations on 'creatinine' and the diazo color reaction should not be relied on in estimating progress or prognosis."

Most observers have noted a lack of relation between the levels of creatinine and urea in cases of renal disease, the chief exception being cases of anuria, e.g., bichloride poisoning. Obviously in such cases both creatinine and urea are being simultaneously and completely retained. The situation is different, however, in cases of nephrosclerosis, chronic glomerulonephritis, intestinal obstruction and the renal complications associated with prostatic hypertrophy and neoplasms of the bladder. In the last mentioned conditions there may be oftentimes a disproportionate increase in the blood urea. Some years ago in following cases at the Cleveland City Hospital in cooperation with Dr. R. O. Bowman and Miss Miriam Dice, it appeared that we had made an interesting observation bearing on this point. It was observed that cases dying from chronic glomerulonephritis showed much higher blood urea values than cases dying with nephrosclerosis with essentially the same values for creatinine, i.e., whereas a case of chronic glomerulonephritis with a blood creatinine of 5 to 10 mg. might have a blood urea nitrogen of 200, a case of nephrosclerosis, with the same range of blood creatinine values, would show a urea nitrogen of only 50 to 70 mg. However, on studying further cases (all diagnoses were confirmed by autopsies) it was found that the above statements were true in only 65 to 75 per cent of the cases.

The Van Slyke urea clearance test is not only an exceedingly valuable diagnostic test of renal insufficiency, but furnishes very valuable prognostic information as well. In this connection, Van Slyke et al.¹¹ conclude: "In patients with diminished renal function the blood urea clearance shows evidence of the diminution sooner than does the blood creatinine content, the blood urea content without relation to the urea excretion, or the phenolsulfonphthalein excretion. The blood urea clearance usually falls below 50 per cent of its normal value before any of the other three values show any abnormality. Only after the blood urea clearance indicates less than 20 per cent of normal renal function are all values for blood urea and creatinine content, and for phenolsulfonphthalein excretion, found outside the limits of normal variation." Bruger and Mosen-thal¹² have also compared the blood creatinine with the urea clearance test and state: "It has been appreciated for a long time that creatinine rises in the blood only when renal function becomes markedly impaired (Chace and Myers, Myers, Fine and Lough, and Myers and Lough). Our results show that normal creatinine values may be found in the blood with as much as 85 per cent of kidney function lost, as measured by the urea clearance test. When the urea clearance has fallen to about 5 per cent of normal and uremia is impending, the creatinine begins to mount in the blood."

High Creatinine Values in Acute Conditions.—Not long after the original publication of Myers and Lough²⁹ in 1915 a number of different workers observed that a blood creatinine in excess of 5 mg. per 100 c.c. did not possess the same unfavorable significance in acute conditions as in chronic nephritis, and presented reports on survival cases, the largest number being cases of bichloride poisoning. Myers and Killian³⁰ reported two acute cases with creatinines of 6.1 and 5.6 mg. respectively with recovery. They also called attention to the fact that occasionally in the acute exacerbation of chronic nephritis the creatinine may exceed 5 mg. for a short period, then decline below this level, rising again

above this level before fatal termination. Occasionally this may occur several times in the course of the disease.

Non-fatal cases of mercury bichloride poisoning with high creatinine values have been reported as follows: Cohen and Bernhard⁵² 9.3 mg., Campbell⁵⁴ 12.5 mg., Killian⁵⁵ two cases, 12.0 and 15.2 mg. respectively, Gatewood and Byfield⁵⁶ 11.3 mg., Muntwyler, Way and Pomerene⁵⁷ two cases, 12.0 and 15.5 mg. respectively and Bowman and Wolpaw⁵⁸ a case of uremia, apparently due to mercuric chloride, 24.3 mg. per 100 c.c. Although recovery in such cases appears to be the exception, rather than the rule, the cases cited indicate that while more than 5 mg. creatinine per 100 c.c. of blood furnishes a grave prognosis in poisoning with mercuric chloride, the prognosis need not necessarily be hopeless.

TABLE II

CASE (L. M.) WITH ANURIA WITH VERY HIGH CREATININE AND RECOVERY⁵⁹

DATE 1922	CHEMICAL BLOOD ANALYSES			
	CREATININE	UREA N	URIC ACID	CO ₂ CAPACITY
	mg.	mg.	mg.	c.c.
Mar. 13		12.1	4.6	
Mar. 27	12.5	62.5		50
Mar. 30	17.0	83.3		32
Mar. 31	23.1	92.0		21
Apr. 2	20.4	98.6		56
Apr. 3	20.0	82.0		82
Apr. 4	21.2	104.0		63
Apr. 10	9.2	56.6		56
Apr. 15	4.4	48.0		63
Apr. 22	3.2	12.9		61
May 4	2.7	9.7		56
July 11		16.0	4.2	

Nephrolithiasis; patient developed anuria following a unilateral pyelogram and displacement of renal calculus; alkali therapy Mar. 31 to Apr. 3, forced fluids through duodenal tube from Mar. 31; passed calculus Apr. 8; discharged May 15.

The highest blood creatinine we have observed with ultimate recovery is given in Table II. This patient (L. M.) developed anuria following an unilateral pyelogram and displacement of renal calculus.⁵⁹ After the blood creatinine had risen to 23.1 mg. and the CO₂ combining power of the plasma had fallen to 21, alkali therapy was started and fluid forced with the aid of a duodenal tube, about 1,000 to 4,000 c.c. of fluid being given daily. It is believed that in this case the blood analyses gave a clear understanding of the condition without which the patient unquestionably would have died.

Selman and Linegar⁶⁰ have reported a very interesting case in which the creatinine rose to 15.4 mg. and the urea nitrogen to 142 mg. per 100 c.c. with ultimate recovery. The patient suffered from severe asthma and shock, the blood pressure being too low to measure with a mercury manometer. The authors suggest the resultant disturbance in blood flow to the kidney as the probable cause of the elevation of the nitrogenous metabolites in the blood. It took about eight days for the blood accumulation to reach its peak, while twelve days were required for its return to normal.

Kilduffe⁶¹ in discussing a case which remained alive for fifteen months with blood creatinine values ranging from 10 to 20 mg. per 100 c.c., made the

following comment regarding recovery cases: "Hypercreatininemia may, with two exceptions, be accepted as of ominous import, the observations of many indicating that a blood creatinine of more than 5 mg. per cent is the rather immediate forerunner of a fatal termination. The exceptions to this clinical rule are: (1) anuria, in which, if the cause can be found and removed, creatinine retention is not of prognostic import, and (2) acute nephritis in which temporary high values may be encountered. With these two exceptions hypercreatininemia always signifies an extreme degree of functional renal insufficiency, the kidney apparently being unable to recover from the damage thus indicated. . . . The usual interpretation regards creatinine values of 2.5 to 3 mg. per cent as suspicious; 3 to 5 mg. per cent as unfavorable, and over 5 mg. per cent as indicative of an early fatal termination. While occasional recoveries in the presence of hypercreatininemia have been reported, these are so exceptional as not to invalidate the general rule."

COMPARISON OF CREATININE WITH OTHER PROGNOSTIC TESTS

In addition to the blood urea, and Van Slyke urea clearance tests already mentioned, and the fact that a sudden and pronounced upturn in the blood uric acid may be an unfavorable prognostic sign several other tests have been suggested which have a grave prognostic omen. These are an elevation of the serum inorganic phosphorus, increases in the amount of indican and strongly positive diazo and xanthoproteic reactions. Most of these become positive slightly later than the creatinine, and therefore are even more grave signs of impending death. However, it appears doubtful if they are any more reliable than the creatinine, and the fact that they become definitely positive a little later can hardly be considered an advantage. Nevertheless, they are excellent confirmatory tests.

Inorganic Phosphorus.—de Wesselow⁶² in particular has emphasized the unfavorable prognostic value of an elevated inorganic phosphorus in nephritis, especially when associated with uremia. He points out that on the average the retention of phosphates and urea run a parallel course, but observes that a confident prognosis can scarcely be ventured upon the estimation of the blood urea alone. He is of the opinion that although nitrogen retention is a valuable prognostic index of the severity of nephritis, nitrogen retention per se is harmless. His special reason for suggesting the use of the inorganic phosphorus as a prognostic test in azotemic nephritis at the time was due to the question which had just been raised as to the accuracy of the creatinine estimation by Benedict.³ He states: "A plea may therefore be uttered for the value of the plasma content in inorganic phosphorus (phosphate) in estimating the probable outcome of a case of azotemic nephritis." He regarded an inorganic phosphorus of 2 to 4 mg. per 100 c.c. of plasma as normal, and above 10 mg. as a grave prognosis.

To secure information on this point, and on the serum calcium, Schmitz, Rohdenburg and Myers⁶³ studied a series of 54 cases with markedly impaired renal function. On the basis of their data it would appear that the values for the creatinine and serum inorganic phosphorus are equally significant, but that the fluctuations in the blood urea are so great as to be much less significant. However, it was the authors' opinion on the basis of following the serial findings

in the individual cases that there was little doubt about the reliability of inorganic phosphorus retention as a prognostic sign in chronic nephritis, but that creatinine is equally reliable and the retention appears to occur appreciably earlier than is the case with the inorganic phosphorus.

Diazo-Color Reaction.—Andrewes,⁶⁴ while carrying out the van den Bergh reaction for bilirubin on blood serum, noted a deviation from the normal color. When alkali was added to the end-product a pink coloration was obtained instead of the green of azobilirubin. This reaction was found to occur only in persons with markedly damaged kidneys. Hewitt⁶⁵ modified the method so as to rule out false positives.

According to Harrison and Broomfield⁶⁶ the substance responsible for the reaction is an indoxyl compound, presumably indican, or possibly, in part, indoxyl glycuronate. It is therefore not surprising that the results obtained with the diazo reaction harmonize with the indican test (see below).

Rabinowitch⁶⁷ has apparently made a more extensive clinical study of this test than anyone else (also gives literature). He observed that when an attempt was made to correlate the positive reactions with the urea content of the blood no correlation was noted. This, he felt, was to be expected, since the prognosis and degree of retention of blood urea are not related. Although Rabinowitch made many hundreds of analyses, and frequently found high blood ureas associated with negative diazo-color reactions, he never found a positive diazo-color reaction associated with a normal blood urea nitrogen. In the case of creatinine Rabinowitch commented: "here there appears to be a relationship," his observations being reproduced in Table III. He noted two cases with creatinine values greater than 5 mg. per 100 c.c. of blood with negative diazo-color reactions, but in these cases the anurias were due to mechanical block (prostatic hypertrophy). The conclusion was drawn that a positive diazo-color reaction is not found in any condition other than severe kidney damage, although a positive reaction may be found in persons with severe kidney damage, and such persons may recover. Such cases include the albuminurias of severe infections, the well-recognized form

TABLE III
RELATIONSHIP BETWEEN POSITIVE DIAZO-COLOR REACTION AND CREATININE
CONTENT OF BLOOD (RABINOWITCH⁶⁷)

BLOOD CREATININE	INCIDENCE OF POSITIVE DIAZO-COLOR REACTION
mg. per 100 c.c.	
-1.50	0
1.51-1.75	0
1.76-2.00	0
2.01-2.25	3
2.26-2.50	5
2.51-2.75	5
2.76-3.00	6
3.01-3.25	8
3.26-3.50	7
3.51-3.75	11
3.76-4.00	14
4.01-4.25	16
4.26-4.50	20
4.51-4.75	15
4.76-5.00	26
5.01-	41

of acute nephritis with pallor, edema, etc., acute exacerbations of chronic nephritis, mechanical obstruction in the urinary flow, surgical lesions of the kidneys with urinary retention, and the anuria of diabetic coma. When acute exacerbations of the disease are excluded, a positive reaction occurring in the course of chronic nephritis indicates an unfavorable prognosis.

Indican.—In 1911 Ohrmayer and Popper⁶⁸ called attention to the fact that indican (indoxyl potassium sulfate) is increased in the blood of patients with uremia. Later Rosenberg⁶⁹ compared the blood indican and creatinine, as noted above, observing that in azotemia urea was elevated first, then creatinine and finally indican. However, he appeared to favor creatinine as a prognostic test, partly because of the greater simplicity in its estimation. A large literature has accumulated on the subject. Among papers which might be mentioned in chronological order are those by Haas,⁷⁰ Monias and Shapiro,⁷¹ Keith and Wakefield,⁷² Sharlitt,⁷³ Polayes and Eckert,⁷⁴ and Kenny and Hubbard.⁷⁵

The normal for the blood indican is generally given as 0.05 to 0.15 mg. per 100 c.c. of plasma or serum. Values of 0.2 mg. are regarded as abnormal but the values may reach 10 mg. per 100 c.c. in severe uremia. Sharlitt⁷³ has described a new method which possesses several desirable features. He regards the normal as less than 0.06 mg. and found values to range from 1.55 to 6.20 mg. in ten pathological cases.

Monias and Shapiro⁷¹ have presented data on over a hundred cases in which urea and creatinine were also determined, and regard the indican as definitely superior to creatinine and urea as a prognostic test in uremia. A study of their data, however, reveals the fact that in the cases in which the urea and creatinine findings were less significant than the indican, the blood analyses were made a month or more before the death of the patient. Keith and Wakefield⁷² regard an elevation of the indican of the blood as somewhat comparable to the elevation of creatinine, and therefore of prognostic and not of diagnostic significance. While indican is increased by intestinal obstruction, chronic suppuration or infections of long standing and hepatic insufficiency, its elevation in the blood is most marked in renal insufficiency. To visualize the comparative significance of data for urea, creatinine, indican and the xanthoproteic reaction, observations made by Kenny and Hubbard are presented in Table IV. The Table includes only cases in which creatinine determinations were made, the data being rearranged according to the magnitude of the creatinine. On the basis of the data on these 13 cases, the findings for creatinine, indican and the xanthoproteic readings harmonize very well. However, in this series, the creatinine findings appear superior to the indican and about equal to the xanthoproteic readings.

Xanthoproteic Reaction.—The xanthoproteic reaction on serum filtrate as a measure of renal insufficiency was introduced by Beecher⁷⁶ in 1924. His observation that the test constitutes a reliable prognostic index in uremia has been confirmed by a number of workers, among whom may be mentioned Rasmussen,⁷⁷ Steen,⁷⁸ Kenny and Hubbard,⁷⁵ and recently Ragins, Kraus and Coe.⁷⁹ The test is a simple one, consisting essentially of carrying out the xanthoproteic reaction on the trichloroacetic acid filtrate of a measured amount of serum and matching the color against a standard bichromate solution.

TABLE IV

CREATININE, UREA N AND INDICAN AND XANTHOPROTEIC TESTS IN RENAL INSUFFICIENCY
REARRANGED ON THE BASIS OF THE MAGNITUDE OF THE CREATININE
(DATA OF KENNY AND HUBBARD⁷⁵)

CASE	CREATININE	UREA N	INDICAN	XANTHOPROTEIC READING	DIAGNOSIS, REMARKS
16	31.6	203	2.9	144	Malignant nephrosclerosis. Nausea, vomiting, died, uremia, no autopsy.
3	20.3	193	2.0	142	Chronic glomerulonephritis. Drowsy; muscle twitching, died, uremia, no autopsy.
18	14.1	244	0.8	153	Chronic glomerulonephritis. Nausea, vomiting, coma, died, uremia, no autopsy.
9	13.0	123	3.5	83	Comatose, uremic frost. Autopsy: Malignant nephrosclerosis, uremia.
2	12.8	148	Tr.	133	Comatose. Autopsy: Carcinoma of cervix, pyelonephritis, uremia.
10	9.0	134	3.0	100	Comatose. Autopsy: Hypertrophied prostate, hydronephrosis, uremia.
1	9.0	106	1.7	90	Pyelonephritis, uremia, died, no autopsy.
5	8.2	95	2.1	111	Tubercular kidney, uremia. Nausea, vomiting, death, no autopsy.
8	5.4	64	2.5	100	Chronic glomerulonephritis. Vomiting, died at home.
4	5.3	109	2.3	181	Vomiting. Autopsy: Combined arteriosclerosis and nephritis.
2	4.5	88	Tr.	50	Hypertrophied prostate, pyelonephritis. Operation and improvement.
3	1.3	40	Neg.	62	Cystotomy, death not due to renal insufficiency.
20	1.2	12	Neg.	15	Convulsions. Death: Acute glomerulonephritis, lobar pneumonia.

The normal range of values has generally been given as 25 to 35, but Ragins et al. believe that a more correct normal range is 0 to 50. They consider that xanthoprotein values over 60 should be considered abnormal in all conditions.

Becher⁸⁰ further reported that in the blood of patients with renal insufficiency there was an increase of aromatic substances—an ether soluble group comprising phenols, cresols, aromatic oxyacids and indoxyl, and a non-ether group containing the amino acids, tyrosine, phenylalanine and tryptophane—and have associated this with the increased xanthoproteic reaction. Later with Koeh⁸¹ he reported markedly increased xanthoproteic values (80 to 250) in patients with uremia and indicated that uremia was less dependent on retention of nitrogenous products than on that of aromatic substances, and that phenol poisoning resembled true uremia. Kenny and Hubbard⁷⁵ feel that the xanthoproteic reaction is of special value in distinguishing between true and pseudo-uremia. They state that in pseudo-uremia, or hypertensive encephalopathy, the symptoms are due to cerebral edema and the xanthoproteic reaction remains low regardless of the urea nitrogen. Ragins et al.⁷⁹ concluded from their data that uremia was the only specific condition giving a significantly increased xanthoproteic reaction, although lysol poisoning must be kept in mind as a possibility. A few illustrative observations from the data of Kenny and Hubbard are given in Table IV and show the comparative value of the test.

RESPONSE TO INTRAVENOUSLY ADMINISTERED CREATININE

In 1923 Major⁵² suggested following the two-hour output of creatinine after the intravenous administration of 0.5 Gm. as a test of renal function. He observed that the normal kidneys responded to the intravenous injection of creatinine by an increased output during the same period of time, while in chronic nephritis the kidneys showed on the average only a slight increase, and in many instances a decrease. This test never came into general use for the probable reason that it required the intravenous administration of an expensive compound in sterile solution.

CREATININE CLEARANCE TEST

The creatinine clearance test introduced by Rehberg has been studied by a number of workers as a test of renal function. Hayman, Halsted and Seyler⁵³ made a careful comparison of this test with the urea clearance test to determine if there was sufficient practical advantage in the creatinine test to compensate for the added technical difficulties. In close agreement with Møller, McIntosh and Van Slyke, they found in a series of about 25 normal individuals that the maximum urea clearance averaged 75 c.c. per minute, while the mean standard urea clearance was 51 c.c. per minute. The mean creatinine clearance on about 60 normal subjects was 148 c.c. per minute. This would appear to indicate that the creatinine is normally eliminated two or three times as readily as the urea. However, Hayman et al. found that in patients with Bright's disease the creatinine and urea clearance tests were generally equally reduced in relation to the average normal. Since the variability of the two tests from the average normal was approximately the same, they concluded that the creatinine clearance test did not possess any practical advantages to compensate for its greater technical difficulty (ingestion of 3 to 5 Gm. of expensive creatinine and the analysis of two blood samples).

DISCUSSION

On the basis of the tests discussed it would appear that the Van Slyke urea clearance test gives the most reliable measure of renal insufficiency, especially in the early stages of renal disease. When definite impairment in renal function has been established the level of the blood urea nitrogen should also be taken into consideration, since this is available from the data required for the calculation of the urea clearance. With a definite elevation of the blood urea nitrogen, the creatinine should also be determined. The author believes that practical experience has conclusively demonstrated the superiority of the blood creatinine over the urea nitrogen as a prognostic sign in cases with markedly impaired renal function. While essentially the same prognosis might be given in many cases on the basis of figures for the urea nitrogen alone, there are a considerable number where the creatinine may be elevated above the critical level without the presence of correspondingly high values for the urea nitrogen. It is on this account that the prognostic significance of an elevated blood creatinine is more dependable than that of urea. Theoretically this finding would seem logical since creatinine is more readily eliminated than urea, is excreted by the human kidney somewhat differently than urea in that the tubules participate, and

furthermore is almost entirely endogenous whereas urea under ordinary conditions of diet is largely exogenous in origin. The statement may be made that the urea clearance test is also a good prognostic test. This is true but with marked retention the changes in the blood creatinine are greater and therefore become more significant. The blood creatinine furthermore is an excellent confirmatory test in cases in which low values for the urea clearance are found. It should also be borne in mind that the urea clearance test requires an accurate urine collection, which is sometimes impossible. The creatinine on the other hand requires simply an analysis of the blood, but it would be well in this case always to include determination of urea as well as creatinine.

While the phenolsulfonphthalein test has proved itself an extremely valuable renal function test, the output of the dye is so low with severe renal impairment as not to adequately reflect the changes at this stage in the disease.

The rise in the level of the inorganic phosphorus in advanced renal disease is an infallible sign of marked impairment in renal function. In the author's opinion, however, it comes a little later than the retention of creatinine. On this account the creatinine determination would appear superior as a prognostic test.

The value of the determination of indican, the diazo reaction and the xanthoproteic reaction have all been amply demonstrated. Many of the investigators who have worked with these tests have also presented parallel figures for the blood creatinine and urea. While the reliability of these special tests has been proved, in the author's opinion these tests have not been shown to have any greater reliability than the blood creatinine. On this account the estimation of the blood creatinine would appear to remain the method of choice on account of greater simplicity as a routine chemical blood determination.

SUMMARY

The prognostic significance of an elevated creatinine has been discussed on the basis of individual observations and those in the literature. The test has also been considered in comparison with the following tests which have also been advocated for the same purpose, viz., the phenolsulfonphthalein test, the blood urea, the Van Slyke urea clearance test and the serum inorganic phosphorus, indican, diazo-color reaction, and xanthoproteic reaction, also the response to intravenously administered creatinine and the creatinine clearance test.

REFERENCES

1. Folin, O.: Laws Governing the Chemical Composition of the Urine, *Am. J. Physiol.* 13: 65, 1905.
2. Borsook, H., and Dubnoff, J. W.: The Formation of Glycoeyamine in Animal Tissues, *J. Biol. Chem.* 138: 389, 1941.
- Borsook, H., Dubnoff, J. W., Lilly, J. C., and Marriott, W.: The Formation of Glycoeyamine in Man and Its Urinary Excretion, *Ibid.* 138: 405, 1941.
3. du Vigneaud, V., Chandler, J. P., Cohn, M., and Brown, G. B.: The Transfer of the Methyl Group From Methionine to Choline and Creatine, *J. Biol. Chem.* 134: 787, 1940.
4. Bloch, K., and Schoenheimer, R.: The Biological Precursors of Creatine, *J. Biol. Chem.* 138: 167, 1941.
5. Fiske, C. H., and Subbarow, Y.: Nature of "Inorganic Phosphate" in Voluntary Muscle, *Science* 65: 401, 1907.
- Idem: The Isolation and Function of Phosphocreatine, *Science* 67: 169, 1928.
- Idem: Phosphocreatine, *J. Biol. Chem.* 81: 629, 1929.

6. Folin, O., and Denis, W.: Protein Metabolism From the Standpoint of Blood and Tissue Analysis. VII. An Interpretation of Creatine and Creatinine in Relation to Animal Metabolism, *J. Biol. Chem.* 17: 493, 1914.
7. Folin, O., and Denis, W.: On the Creatinine and Creatine Content of Blood, *J. Biol. Chem.* 17: 487, 1914.
8. Neuhauer, O.: Verwendung von Kreatinin zur Prüfung der Nierentunktion, *München. med. Wchnschr.* 61: 857, 1914.
9. Myers, V. C., and Fine, M. S.: A Note on the Retention in the Blood of Uric Acid and Creatinine in the Uremic Type of Nephritis, *Proc. Soc. Exper. Biol. & Med.* 11: 132, 1914.
10. Shaffer, P. A.: Observations on Creatine and Creatinine, *J. Biol. Chem.* 18: 525, 1914.
11. Shaffer, P. A., and Reinoso, E. A.: Note on the Determination of Kreatinin. Do Muscle and Blood Contain Kreatinin? *J. Biol. Chem.* 7: (proc.) xiii and xxx, 1910.
12. Folin, O.: On the Determination of Creatinine and Creatine in Blood, Milk and Tissues, *J. Biol. Chem.* 17: 475, 1914.
13. Myers, V. C., and Fine, M. S.: Chemical Composition of the Blood in Health and Disease. III. Creatinine and Creatine, New York, 1915, pp. 19-22.
14. Hunter, A., and Campbell, W. R.: The Probable Accuracy, in Whole Blood and Plasma, of Colorimetric Determinations of Creatinine and Creatine, *J. Biol. Chem.* 32: 413, 1917.
15. Folin, O., and Svedberg, A.: Diffusible Non-Protein Constituents of Blood and Their Distribution Between Plasma and Corpuscles, *J. Biol. Chem.* 88: 715, 1930.
16. Behre, J. A., and Benedict, S. R.: Studies in Creatine and Creatinine Metabolism. IV. On the Question of the Occurrence of Creatinine and Creatine in Blood, *J. Biol. Chem.* 52: 11, 1922.
17. Benedict, S. R., and Behre, J. A.: Some Applications of a New Color Reaction for Creatinase, *J. Biol. Chem.* 114: 515, 1936.
18. Gaehler, O. H.: The Apparent Creatinine of Serum and Laked Blood Filtrates, *J. Biol. Chem.* 117: 397, 1937.
19. Behre, J. A., and Benedict, S. R.: Experiments on the Precipitation of Creatinine Rubidium Picrate From Blond Plasma Filtrates, *J. Biol. Chem.* 117: 415, 1937.
20. Folin, O.: Non-Protein Nitrogen of Blood in Health and Disease, *Physiol. Rev.* 2: 460, 1922.
21. Hayman, J. M., Jr., Johnston, S. M., and Bender, J. A.: On the Presence of Creatinine in Blood, *J. Biol. Chem.* 108: 675, 1935.
22. Danielson, I. S.: On the Presence of Creatinine in Blood, *J. Biol. Chem.* 113: 181, 1936.
23. Miller, B. F., and Dubos, R.: Studies on the Presence of Creatinine in Human Blood. Determination by a Specific, Enzymatic Method of the Creatinine Content of Blood and Urine From Normal and Nephritic Individuals, *J. Biol. Chem.* 121: 447, 457, 1937.
24. Gaehler, O. H., and Ketch, A. K.: On the Nature of Blood Creatinine, *J. Biol. Chem.* 76: 337, 1928.
25. Gaehler, O. H.: Further Studies on Blood Creatinine, *J. Biol. Chem.* 89: 451, 1930.
26. Gaehler, O. H.: The Apparent Creatinine of Serum and Laked Blood Filtrates, *J. Biol. Chem.* 117: 397, 1936.
27. Gaehler, O. H., and Alhott, L. D., Jr.: Isolation of Creatinine from Serum Ultrafiltrates, *J. Biol. Chem.* 123: 119, 1930.
28. Goudsmit, A., Jr.: On the Origin of Urinary Creatinine, *J. Biol. Chem.* 115: 613, 1936.
29. Myers, V. C., and Lough, W. G.: The Creatinine of the Blood in Nephritis. Its Diagnostic Value, *Arch. Int. Med.* 16: 536, 1915.
30. Chace, A. F., and Myers, V. C.: The Value of Recent Laboratory Tests in the Diagnosis and Treatment of Nephritis, With Special Reference to the Chemical Examination of the Blood, *J. A. M. A.* 67: 929, 1916.
31. Mayrs, E. B.: The Relative Excretion of Urea and Some Other Constituents of the Urine, *J. Physiol.* 56: 58, 1922.
32. Rehberg, P. B.: Studies on Kidney Function. I. Rate of Filtration and Reabsorption in the Human Kidney. II. The Excretion of Urea and Chlorides Analyzed According to a Modified Reabsorption Theory, *Biochem. J.* 20: 447, 1926.
33. MacKay, E. M., and Cockrill, J. R.: The Regulation of Renal Activity. XII. The Relation of the Rate of Creatinine Excretion in the Urine to the Plasma Concentration, *Am. J. Physiol.* 94: 220, 1930.
34. Copé, C. L.: The Excretion of Creatinine by the Human Kidney in Health and in Nephritis, *Quart. J. Med.* 24: 567, 1931.
35. Berglund, H., and Frisk, A. R.: Uric Acid Elimination in Man, *Acta med. Scandinav.* 86: 233, 1935.
36. Myers, V. C., and Killian, J. A.: The Prognostic Value of the Creatinine of the Blood in Nephritis, *Ann. J. M. Sc.* 157: 674, 1919.

37. Myers, V. C.: *Practical Chemical Analysis of Blood*, ed. 2, St. Louis, 1924, The C. V. Mosby Company, p. 42.
38. Richards, A. N.: *Physiology of the Kidney*, Bull. New York Acad. Med. 14: 5, 1938.
39. Rosenberg, M.: Ueber Hyperkreatininämie der Nephritiker und ihre prognostische Bedeutung, München. med. Wchnschr. 63: 928, 1916.
40. Feigl, J.: Über das Vorkommen von Kreatinin und Kreatin im Blute bei Gesunden und Kranken. I, Biochem. Ztschr. 81: 14, 1917.
41. Rabinowitch, I. M.: The Prognostic Value of the Study of the Blood Creatinine, Canad. M. A. J. 11: 320, 1921.
42. Moreau, E., and Diamant, J.: Diagnostic et pronostic des néphrities urémigènes par le dosage clinique de la créatinine dans le sang, Progrès méd. 38: 341, 1923.
43. Jeanbrau, E., and Cristol, P.: Valeur pronostique de l'hypercreatininémie dans néphrites azotémiques, Compt. rend. Soc. de biol. 88: 594, 1923.
44. Feinblatt, H. M.: Creatininemia: Based Upon a Study of Fifteen Hundred Blood Analyses, Am. J. M. Sc. 166: 249, 1923.
45. Hubbard, R. S.: Urea and Creatinine Concentration in Blood: A Statistical Study, Proc. Soc. Exper. Biol. & Med. 25: 261, 1928.
46. Cantarow, A., and Davis, R. C.: Blood Nonprotein Nitrogen and Creatinine in Nephritis and Prostatic Obstruction, J. LAB. & CLIN. MED. 18: 502, 1933.
47. Warter, J.: La rétention de la créatinine et son importance au cours des néphrites, Rev. de méd., Par. 51: 391, 1934.
48. Popper, H., Mandel, E., and Mayer, H.: Die diagnostische Bedeutung der Plasma-kreatininbestimmung, Ztschr. f. klin. Med. 133: 56, 1937.
49. Peters, J. P., and Van Slyke, D. D.: Quantitative Clinical Chemistry. I. Interpretations, Baltimore, 1932, Williams & Wilkins Co., p. 461.
50. Patch, F. S., and Rabinowitch, I. M.: Urea and Creatinine Content of the Blood in Renal Disease. A Statistical Analysis of Five Thousand Observations, J. A. M. A. 90: 1092, 1928.
51. Van Slyke, D. D., McIntosh, J. F., Möller, E., Hannon, R. R., and Johnson, C.: Studies on Urea Excretion. VI. Comparison of the Blood Urea Clearance With Certain Other Measures of Renal Function, J. Clin. Investigation 8: 357, 1930.
52. Bruger, M., and Mosenthal, H. O.: Urea Clearance Test as an Index of Renal Function. IV. The Urea Clearance Test in Relation to Other Tests and Measures of Renal Function, Arch. Int. Med. 50: 556, 1932.
53. Cohen, J., and Bernhard, A.: A Case of Mercurial Poisoning With Recovery, J. A. M. A. 66: 1019, 1916.
54. Campbell, W. R.: Observations on Acute Mercuric Chloride Nephrosis, With a Report of Two Cases, Arch. Int. Med. 20: 919, 1917.
55. Killian, J. A.: A Note on the Blood Chlorides in Mercuric Chloride Nephrosis, J. LAB. & CLIN. MED. 7: 129, 1921.
56. Gatewood, L. C., and Byfield, A. F.: A Clinical Report on Cases of Mercuric Chloride Poisoning, Arch. Int. Med. 32: 456, 1923.
57. Muntwyler, E., Way, C. T., and Pomerene, E.: The Acid-Base Balance in Pathologic Conditions. III. Serum Electrolyte Changes in Acute Mercuric Chloride Poisoning, Arch. Int. Med. 53: 885, 1934.
58. Bowman, R. O., and Wolpaw, S.: Marked Uremia With Recovery; Report of a Case, Ann. Int. Med. 11: 209, 1937.
59. McCarthy, J. F., Killian, J. A., and Chace, A. F.: Reflex Anuria. Report of a Case With Unusual Blood Chemistry Findings and an Unusual Application of the Duodenal Tube, J. A. M. A. 70: 1043, 1923.
60. Selman, J. J., and Linegar, C. R.: A Case of Marked Creatininemia With Recovery, J. LAB. & CLIN. MED. 18: 1032, 1933.
61. Kilduffe, R. A., and Salasin, S. L.: Sustained Hypercreatininemia With Delayed Fatal Termination. Case Report, J. M. Soc. New Jersey 29: 42, 1932.
62. de Wesselow, O. L. V.: The Immediate Prognosis in Nephritis, With Some Remarks on Uraemia, Lancet II: 163, 1923.
63. Schmitz, H. W., Rohdenburg, E. L., and Myers, V. C.: The Inorganic Phosphorus and Calcium of the Blood in Nephritis, Arch. Int. Med. 37: 233, 1926.
64. Andrewes, L. F.: The Diazo Reaction in Uraemic Sera, Biochem. J. 19: 171, 1929.
65. Hewitt, C. H.: An Unexplained Diazo-Color Reaction in Uraemic Sera, Lancet I: 590, 1924.
66. Harrison, G. A., and Broomfield, R. J.: The Cause of Andrewes's Diazo-Test for Renal Insufficiency, Biochem. J. 22: 43, 1928.
67. Rabinowitch, I. M.: The Diazo Color Reaction Found in Uremia, Arch. Int. Med. 45: 282, 1930.
68. Obermayer, F., and Popper, H.: Ueber Urämie, Ztschr. f. klin. Med. 72: 332, 1911.
69. Rosenberg M.: Beiträge zur Pathochemie des Reststickstoffs bei Nierenkranken. I. Indican und Kreatinin, Arch. f. exper. Path. u. Pharmacol. 86: 15, 1920.
70. Haas, G.: Das Blutindican und seine praktisch diagnostische Bedeutung, München. med. Wchnschr. 64: 1363, 1917.

71. Monias, B. L., and Shapiro, P.: The Value of the Indican Determination in the Blood in Cases of Renal Insufficiency, *Arch. Int. Med.* 45: 573, 1939.
72. Keith, N. M., and Winkfield, E. G.: Indican in the Blood: A Test of Renal Function, *M. Clin. North America* 16: 1401, 1933.
73. Sharlit, H.: A Method for the Quantitative Estimation of Indoxyl Compounds in Blood, *J. Biol. Chem.* 104: 115, 1934.
74. Polayes, S. H., and Eckert, E. A.: Observations on the Indican Test of Blood and Urine in Renal Insufficiency, *J. LAB. & CLIN. MED.* 20: 681, 1935.
75. Kenny, F. E., and Hubbard, R. S.: Xanthoproteic and Indican Studies in the Blood in Renal Insufficiency, *Am. J. Clin. Path.* 9: 465, 1939.
76. Becher, E.: Ueber eine neue einfache Methode zur Feststellung der Niereninsuffizienz in Blut, *München. med. Wchnschr.* 71: 1611, 1924.
77. Rasmussen, H.: Xanthoproteic Reaction in Blood as Test of Renal Function, *Acta med. Scandinav.* 86: 302, 1935.
78. Steen, W. B.: Value of Blood Xanthoproteic Reaction in Diagnosis and Prognosis, *J. LAB. & CLIN. MED.* 22: 825, 1937.
79. Ragins, O. B., Kruss, I., and Coe, J. C.: The Significance of the Xanthoprotein Reaction in Renal Insufficiency and Other Clinical Conditions, *J. LAB. & CLIN. MED.* 27: 201, 1941.
80. Becher, E.: Über das Vorkommen aromatischer Gruppen in entweißtem Blut, Körperflüssigkeiten und Geweben, nachgewiesen am Ausfall der Xanthoproteinreaktion, *München. med. Wchnschr.* 71: 1677, 1924.
81. Becher, E., and Koch, F.: Über die pathogenetischen Beziehungen zwischen echter Urämie und den bei Niereninsuffizienz im Blut retinierten Substanzen, *Deutsch. Arch. f. klin. Med.* 148: 78, 1923.
82. Major, R. H.: The Use of Creatinin as a Test of Renal Function, *J. A. M. A.* 80: 384, 1923.
- Idem: Creatinin Test for Renal Function, *Arch. Int. Med.* 33: 69, 1924.
83. Hayman, J. M., Jr., Halsted, J. A., and Seyler, L. G.: A Comparison of the Creatinine and Urea Clearance Tests for Kidney Function, *J. Clin. Investigation* 12: 861, 1933.
84. Arkin, A., Popper, H., and Goldberg, F. A.: Plasma Creatinine Determination as Test of Low Grade Kidney Damage, *Ann. Int. Med.* 15: 700, 1941.

37. Myers, V. C.: *Practical Chemical Analysis of Blood*, ed. 2, St. Louis, 1924, The C. V. Mosby Company, p. 42.
38. Richards, A. N.: *Physiology of the Kidney*, Bull. New York Acad. Med. 14: 5, 1938.
39. Rosenberg, M.: Ueber Hyperkreatininämie der Nephritiker und ihre prognostische Bedeutung, München. med. Wchnschr. 63: 928, 1916.
40. Feigl, J.: Über das Vorkommen von Kreatinin und Kreatin im Blute bei Gesunden und Kranken. I, Biochem. Ztschr. 81: 14, 1917.
41. Rabinowitch, I. M.: The Prognostic Value of the Study of the Blood Creatinine, Canad. M. A. J. 11: 320, 1921.
42. Moreau, E., and Diamant, J.: Diagnostic et pronostic des néphrites urémigènes par le dosage clinique de la créatinine dans le sang, Progrès méd. 38: 341, 1923.
43. Jeanbraun, E., and Cristol, P.: Valeur pronostique de l'hypercreatininémie dans néphrites azotémiques, Compt. rend. Soc. de biol. 88: 594, 1923.
44. Feinblatt, H. M.: Creatininemia: Based Upon a Study of Fifteen Hundred Blood Analyses, Am. J. M. Sc. 166: 249, 1923.
45. Hubbard, R. S.: Urea and Creatinine Concentration in Blood: A Statistical Study, Proc. Soc. Exper. Biol. & Med. 25: 261, 1928.
46. Cantarow, A., and Davis, R. C.: Blood Nonprotein Nitrogen and Creatinine in Nephritis and Prostatic Obstruction, J. LAB. & CLIN. MED. 18: 502, 1933.
47. Warter, J.: La rétention de la créatinine et son importance au cours des néphrites, Rev. de méd., Par. 51: 391, 1934.
48. Popper, H., Mandel, E., and Mayer, H.: Die diagnostische Bedeutung der Plasma-kreatininbestimmung, Ztschr. f. klin. Med. 133: 56, 1937.
49. Peters, J. P., and Van Slyke, D. D.: *Quantitative Clinical Chemistry. I. Interpretations*, Baltimore, 1932, Williams & Wilkins Co., p. 461.
50. Patch, F. S., and Rabinowitch, I. M.: Urea and Creatinine Content of the Blood in Renal Disease. A Statistical Analysis of Five Thousand Observations, J. A. M. A. 90: 1092, 1928.
51. Van Slyke, D. D., McIntosh, J. F., Möller, E., Hannon, R. R., and Johnson, C.: Studies on Urea Excretion. VI. Comparison of the Blood Urea Clearance With Certain Other Measures of Renal Function, J. Clin. Investigation 8: 357, 1930.
52. Bruger, M., and Mosenthal, H. O.: Urea Clearance Test as an Index of Renal Function. IV. The Urea Clearance Test in Relation to Other Tests and Measures of Renal Function, Arch. Int. Med. 50: 556, 1932.
53. Cohen, J., and Bernhard, A.: A Case of Mercuric Poisoning With Recovery, J. A. M. A. 66: 1019, 1916.
54. Campbell, W. R.: Observations on Acute Mercuric Chloride Nephrosis, With a Report of Two Cases, Arch. Int. Med. 20: 919, 1917.
55. Killian, J. A.: A Note on the Blood Chlorides in Mercuric Chloride Nephrosis, J. LAB. & CLIN. MED. 7: 129, 1921.
56. Gatewood, L. C., and Byfield, A. F.: A Clinical Report on Cases of Mercuric Chloride Poisoning, Arch. Int. Med. 32: 456, 1923.
57. Muntwyler, E., Way, C. T., and Pomerene, E.: The Acid-Base Balance in Pathologic Conditions. III. Serum Electrolyte Changes in Acute Mercuric Chloride Poisoning, Arch. Int. Med. 53: 885, 1934.
58. Bowman, R. O., and Wolpaw, S.: Marked Uremia With Recovery; Report of a Case, Ann. Int. Med. 11: 209, 1937.
59. McCarthy, J. F., Killian, J. A., and Chace, A. F.: Reflex Anuria. Report of a Case With Unique Blood Chemistry Findings and an Unusual Application of the Duodenal Tube, J. A. M. A. 70: 1043, 1923.
60. Selman, J. J., and Linegar, C. R.: A Case of Marked Creatininemia With Recovery, J. LAB. & CLIN. MED. 18: 1032, 1933.
61. Kilduffe, R. A., and Salasin, S. L.: Sustained Hypercreatininemia With Delayed Fatal Termination. Case Report, J. M. Soc. New Jersey 29: 42, 1932.
62. de Wesselow, O. L. V.: The Immediate Prognosis in Nephritis, With Some Remarks on Uraemia, Lancet II: 163, 1923.
63. Schmitz, H. W., Rohdenburg, E. L., and Myers, V. C.: The Inorganic Phosphorus and Calcium of the Blood in Nephritis, Arch. Int. Med. 37: 233, 1926.
64. Andrewes, L. F.: The Diazo Reaction in Uraemic Sera, Biochem. J. 19: 171, 1929.
65. Hewitt, C. H.: An Unexplained Diazo-Color Reaction in Uraemic Sera, Lancet I: 590, 1924.
66. Harrison, G. A., and Broomfield, R. J.: The Cause of Andrewes's Diazo-Test for Renal Insufficiency, Biochem. J. 22: 43, 1928.
67. Rabinowitch, I. M.: The Diazo Color Reaction Found in Uremia, Arch. Int. Med. 45: 282, 1930.
68. Obermayer, F., and Popper, H.: Ueber Urämie, Ztschr. f. klin. Med. 72: 332, 1911.
69. Rosenberg M.: Beiträge zur Pathochemie des Reststickstoffs bei Nierenkrankheiten. I. Indican und Kreatinin, Arch. f. exper. Path. u. Pharmacol. 86: 15, 1920.
70. Haas, G.: Das Blutindican und seine praktische diagnostische Bedeutung, München. med. Wchnschr. 64: 1363, 1917.

erythrocytes. The changes occurred chronologically in the order as listed. One of the animals died following a dosage of 1.04 millicuries per pound of body weight; its bone marrow was aplastic. Scott and Lawrence emphasize, however, that the tolerance levels were about ten times those used therapeutically. Several of the reports describing clinical experience with the use of radioactive phosphorus contain examples of toxic effects on the bone marrow. Kenney and Craver mention one patient with lymphosarcoma who developed an aplastic anemia under treatment, one who developed a temporary anemia, and several who had transient leucopenia.¹⁶ Erf, Tuttle, and Lawrence⁵ show that several of their patients with lymphatic leukemia who were treated with radioactive phosphorus showed significant lowering of the platelet count while they were apparently improving otherwise; in no instances were hemorrhagic manifestations reported. In one of eleven patients with polycythemia, Erf and Jones noted a fall in platelets from 180,000 to 64,000 per cubic millimeter.¹¹ Kenney reported that in one of his patients with acute lymphatic leukemia the terminal appearance of the bone marrow was one of extreme aplasia with multiple hemorrhages.⁷ He stated that: "The marrow changes were more severe than those ever seen in this institution (Memorial Hospital, New York City) in terminal leukemia not treated with radioactive phosphorus." Diamond and Warren, furthermore, observed that several children with acute leukemia whom they treated with radioactive phosphorus had aplastic marrows at autopsy.¹⁷

The diagnoses established for the 100 patients included in this study were polycythemia vera, 18; chronic myelogenous leukemia, 25; chronic lymphatic leukemia, 22; leucosarcoma, 13;¹⁸ monocytic leukemia, 4; plasma cell myeloma, 4; lymphosarcoma, 2; reticulum cell sarcoma, 3, Hodgkin's disease, 6; giant follicular lymphoblastoma, 1. Patients were accepted for treatment irrespective of the stage of their disease or the previous treatment they had received. They were refused only when their residences were so far from St. Louis as to preclude frequent visits to the clinic.

MATERIALS AND METHODS

The radioactive phosphorus was prepared in the medical cyclotron of the Mallinckrodt Institute* by bombardment of red phosphorus by 12 million volt deuterons. The phosphorus was then synthesized into its dibasic sodium salt, according to the procedure used by Kamen,¹⁹ and was dissolved in enough water to make an isotonic solution (15 mg. per cubic centimeter).† The radio-

*Built with funds provided by the Rockefeller Foundation

†The details of the method are as follows:

1. The phosphorus from the target is washed first with water, then with aqua regia.
2. 100 c.c. aqua regia are added and the mixture is boiled until syrupy to oxidize the P to P_2O_5 .
3. Small amounts of conc. HCL are added and heating is continued until no more brown fumes are given off (indicating that nitric acid has gone). There is no danger of evaporating off the phosphorus pentoxide as there is enough copper present to form copper phosphate.
4. The digest is cooled and diluted with water to about 200 c.c.
5. H_2S is bubbled through the solution for from one to two minutes to precipitate the copper. CuS suspension will coagulate with heat if there is no nitric acid present. The liquid is freed of precipitate by filtration through a Buchner funnel.
6. The filtrate is evaporated carefully so as not to lose the P_2O_5 . As long as the liquid continues to fizz, HCL is present; when white fumes appear, the evaporation has been carried too far. The phosphorus is now in the form of H_3PO_4 .
7. It is neutralized to phenol red with 1N NaOH.
8. Any copper precipitated as copper hydroxide is removed by filtration and the solution is diluted to isotonic concentration. The amount of phosphorus present can be calculated from the amount of NaOH required to neutralize the phosphoric acid. Actually 1.85 c.c. of water are required in the final dilution per 1 c.c. 1N NaOH used in the titration. The isotonic solution of Na_2HPO_4 contains 0.15 mg. of the salt per cubic centimeter.

CLINICAL AND EXPERIMENTAL

HEMATOLOGIC COMPLICATIONS OF THERAPY WITH RADIOACTIVE PHOSPHORUS

LOUIS A. HEMPELMANN, JR., M.D., EDWARD H. REINHARD, M.D.,
CARL V. MOORE, M.D., OLGA S. BIERBAUM, B.S., AND
SHERWOOD MOORE, M.D., ST. LOUIS, MO.

DURING the past two and one-half years, 116 patients with various types of hematologic dyscrasias have been treated at the Washington University School of Medicine with radioactive phosphorus. Of these, 100 have been observed for a sufficiently long period of time to permit evaluation of the therapy. The results in general give weight to the conclusions of other investigators that radioactive phosphorus is a valuable therapeutic agent in the management of polycythemia vera and the chronic forms of leukemia.¹⁻¹¹ Effectiveness in patients with most acute leukemias, Hodgkin's disease, reticulum cell sarcoma, and lymphosarcoma have been disappointing. A detailed analysis of these therapeutic results will be made elsewhere. The primary purpose of this communication is to call attention to the frequency with which anemia, leucopenia, and thrombocytopenia developed in the patients under treatment. In most instances, there was excellent evidence that these complications were induced by the therapy and were not manifestations of the disease per se. They frequently appeared weeks or several months after the last injection of radioactive phosphorus had been given. In the patients with polycythemia, they were often produced by amounts of radioactivity significantly less than those used in other clinics.^{10, 11}

Since it is well known that excessive exposure to radium and x-ray causes toxic effects on the bone marrow, these hematologic complications are not unexpected. With x-ray and radium, however, at least when used in therapeutic doses, the changes induced in the peripheral blood appear promptly.^{12, 13} As indicated above, this is frequently not true for radioactive phosphorus, presumably because with its half-life of 14.3 days it continuously emits radiant energy for weeks after administration. It is not surprising, therefore, that the appearance of toxic effects may be delayed for many weeks. While no emphasis has previously been given to this possibility, Scott and Cook¹⁴ and Scott and Lawrence¹⁵ have studied the effect of radioactive phosphorus on the blood of growing chicks and of monkeys. In chicks, with the dosages used, the polymorphonuclear leucocytes became greatly reduced in numbers, but there were no significant changes in the other formed elements. In monkeys, radioactive phosphorus lowered the absolute numbers of lymphocytes, granulocytes, and

From the Edward Mallinckrodt Institute of Radiology and the Department of Internal Medicine, Washington University School of Medicine.

The expenses of this study were defrayed in part by a grant from the Commonwealth Fund.

Presented in part before the 16th Annual Meeting of the Central Society for Clinical Research, Chicago, Nov. 5, 1943.

Received for publication, Aug. 2, 1944.

Diagnoses were established by study of the peripheral blood and sternal marrow removed by aspiration and by biopsy of lymph nodes and bone marrow. All eighteen patients with polycythemia vera had palpable spleens and were shown to have an increase in the total circulating blood volume. In addition, clinical study proved the absence of pulmonary or circulatory factors which could cause secondary polycythemia. In a few instances the degree of saturation of arterial blood with oxygen was measured.

RESULTS

The incidence, among these patients, of thrombocytopenia, leucopenia, and anemia is summarized in Table I. Sometimes two or all three changes were observed in the same individual. If so, the pattern most frequently seen was first a fall in the white count, then in the platelets, and lastly in the erythrocyte level. It should be emphasized, however, that the platelets occasionally fell to very low levels even in patients who did not develop a leucopenia or an anemia.

A. Thrombocytopenia.—Perhaps the most striking complication was the thrombocytopenia. The platelet count fell to below 100,000 per cubic millimeter in forty-four instances and to below 50,000 in thirty-three of these. These figures do not include any patients in whom the fall in platelets was obviously a manifestation of the dyscrasia. It frequently developed, in patients with leukemia, for instance, several weeks or months after the white cell count had approached normal and most of the abnormal leucocytes had disappeared from the peripheral blood. In some cases, the thrombocytopenia occurred even after therapy had been discontinued. An excellent example is provided by the case of J. M., a man with chronic myelogenous leukemia (Fig. 1). The remission induced in him was the best ever obtained in this series of cases. He received only 10.073 millicuries of radioactive phosphorus in a period of fifty-two days, during which time his white cell count fell from 111,000 to 10,500 cells per cubic millimeter. There was striking clinical improvement with an attendant decrease in the size of his spleen. Yet four weeks after the last injection of radioactive phosphorus and six weeks after the white cell count had returned to a normal range and myelocytes had almost entirely disappeared from the differential, the platelets began to fall. When they reached 40,000 per cubic millimeter, it was necessary to hospitalize the patient because he was bleeding from his gums and complained of overwhelming weakness. There was no fever. Two transfusions were given, and the platelets slowly returned to normal levels. The thrombocytopenia in this instance could hardly be related to an exacerbation of the disease.

Thrombocytopenia is extremely rare in polycythemia vera. For that reason, its occurrence in N. W., a 59-year-old woman with polycythemia vera, after she had received only 7.56 millicuries of radioactive phosphorus, is particularly interesting (Fig. 2). The 7.56 millicuries was all given orally in two doses on succeeding days. This was one of the very few cases in which the phosphorus solution was given by mouth; and, if it is assumed that 75 per cent was absorbed from the gastrointestinal tract,²² this amount would be equivalent to 5.67 millicuries by intravenous injection. This woman returned to the hospital before her scheduled appointment because of moderately ex-

activity of the phosphate solution was determined by means of a Lauritson electroscope calibrated with a uranium standard. The initial activity of a freshly prepared solution usually varied between 300 and 400 microcuries per cubic centimeter. The solution of radioactive phosphorus was used during a period of weeks until its strength had decayed to 30 to 50 microcuries per cubic centimeter.

The plan of treatment for all the diseases except polycythemia closely followed the "fractional method" of therapy outlined by Low-Beer, Lawrence, and Stone.¹⁰ This refers to small frequent intravenous injections of radioactive phosphorus. In only a very few instances was the material given by mouth. The size of each dose given parenterally varied between 100 and 2,000 microcuries. Patients were usually treated two or three times a week at first. As the white blood cell count either approached normal in the patients with leukemia or became subnormal in the cases where the white blood count was not elevated at the onset of treatment, the dosage was decreased and the time interval between treatments was lengthened. Patients with polycythemia were given larger quantities of radioactive phosphorus at much less frequent intervals.

Therapy was governed principally by the changes in the peripheral blood. An attempt was made in each case to restore the cytology of the blood to as near normal as possible and to maintain this effect. Considerable individualization of treatment was necessary because of the great variation in the response shown by different persons. The remissions induced in most patients were sufficiently good to permit interruption of therapy for a period of months. In a few instances it seemed desirable to continue regular injections of the radioactive phosphorus at intervals of several weeks. More active therapy was reinstituted whenever the changes in the peripheral blood indicated that the remission had ended, even though no symptoms developed at these times.

In the cases of lymphosarcoma, Hodgkin's disease, reticulum cell sarcoma, multiple myeloma, and giant follicular lymphoblastoma, where the white blood cell count was not elevated, regulation of therapy was more difficult. Administration of radioactive phosphorus was continued in these instances until changes in the peripheral blood gave warning of a depression of bone marrow activity. In some patients serial bone marrow aspirations were done to aid in regulating therapy. Patients with subleukemic leukemia were given amounts that were comparable with those required to regulate the subjects with elevated counts.

On each visit to the clinic, the patient was examined and the following laboratory data were obtained: total erythrocyte and white blood cell counts, hemoglobin, reticulocyte level, platelet count, and a blood film examination. While fixed cover slip preparations were made for permanent records, the differentials were made routinely with the supravital technique. The blood-counting equipment had all been standardized by the U. S. Bureau of Standards. Hemoglobin determinations were made by the Evelyn oxyhemoglobin method²⁰ on an Evelyn photometer that had been standardized by the simultaneous determinations of the oxygen-combining power. Reticulocyte and platelet counts were done with the Dameshek method.²¹

Diagnoses were established by study of the peripheral blood and sternal marrow removed by aspiration and by biopsy of lymph nodes and bone marrow. All eighteen patients with polycythemia vera had palpable spleens and were shown to have an increase in the total circulating blood volume. In addition, clinical study proved the absence of pulmonary or circulatory factors which could cause secondary polycythemia. In a few instances the degree of saturation of arterial blood with oxygen was measured.

RESULTS

The incidence, among these patients, of thrombocytopenia, leucopenia, and anemia is summarized in Table I. Sometimes two or all three changes were observed in the same individual. If so, the pattern most frequently seen was first a fall in the white count, then in the platelets, and lastly in the erythrocyte level. It should be emphasized, however, that the platelets occasionally fell to very low levels even in patients who did not develop a leucopenia or an anemia.

A. Thrombocytopenia.—Perhaps the most striking complication was the thrombocytopenia. The platelet count fell to below 100,000 per cubic millimeter in forty-four instances and to below 50,000 in thirty-three of these. These figures do not include any patients in whom the fall in platelets was obviously a manifestation of the dyscrasia. It frequently developed, in patients with leukemia, for instance, several weeks or months after the white cell count had approached normal and most of the abnormal leucocytes had disappeared from the peripheral blood. In some cases, the thrombocytopenia occurred even after therapy had been discontinued. An excellent example is provided by the case of J. M., a man with chronic myelogenous leukemia (Fig. 1). The remission induced in him was the best ever obtained in this series of cases. He received only 10.073 millicuries of radioactive phosphorus in a period of fifty-two days, during which time his white cell count fell from 111,000 to 10,500 cells per cubic millimeter. There was striking clinical improvement with an attendant decrease in the size of his spleen. Yet four weeks after the last injection of radioactive phosphorus and six weeks after the white cell count had returned to a normal range and myelocytes had almost entirely disappeared from the differential, the platelets began to fall. When they reached 40,000 per cubic millimeter, it was necessary to hospitalize the patient because he was bleeding from his gums and complained of overwhelming weakness. There was no fever. Two transfusions were given, and the platelets slowly returned to normal levels. The thrombocytopenia in this instance could hardly be related to an exacerbation of the disease.

Thrombocytopenia is extremely rare in polycythemia vera. For that reason, its occurrence in N. W., a 59-year-old woman with polycythemia vera, after she had received only 7.56 millicuries of radioactive phosphorus, is particularly interesting (Fig. 2). The 7.56 millicuries was all given orally in two doses on succeeding days. This was one of the very few cases in which the phosphorus solution was given by mouth; and, if it is assumed that 75 per cent was absorbed from the gastrointestinal tract,²² this amount would be equivalent to 5.67 millicuries by intravenous injection. This woman returned to the hospital before her scheduled appointment because of moderately ex-

J.M., 58 years old Chronic Myelogenous Leukemia

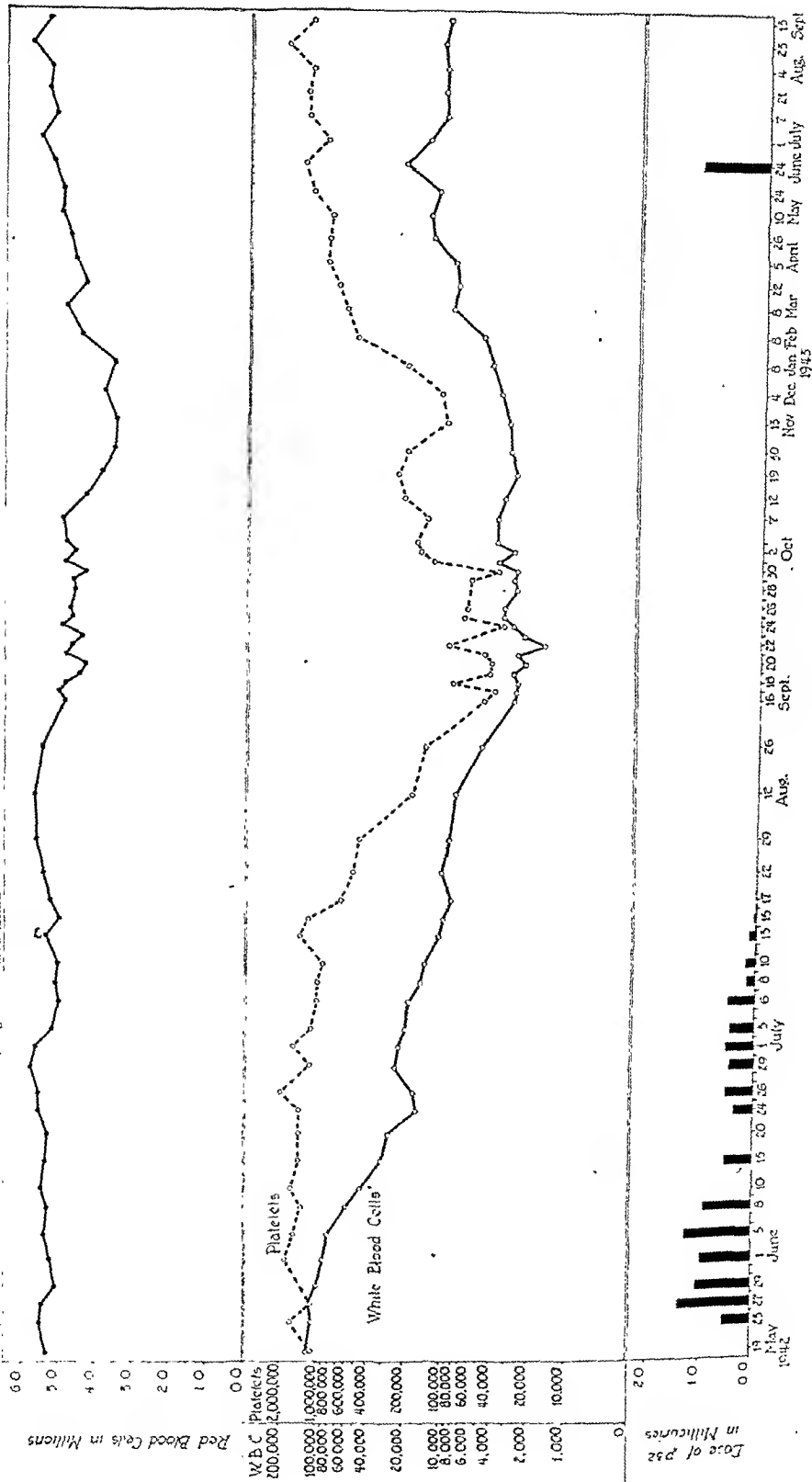


Fig. 1.—For legend see opposite page.

Fig. 1
THROMBOCYTOPENIA AND LEUCOPENIA IN A PATIENT WITH CHRONIC MYELOGENOUS LEUKEMIA FOLLOWING THERAPY WITH P₃₂
DIFFERENTIAL COUNTS

DATE	TOSIN.	BASO.	MYELOCYTES				STAB	SEG.	L.	M.
			A	B	C	JUVENILES				
5/25/42	0	9	0	0	22	22	13	26	2	4
6/8/42	1	12	0	2	4	22	21	34	9	2
7/6/42	0	5	0	0	6	7	6	60	9	7
8/12/42	1	2	0	0	1	0	1	73	14	8
9/22/42	2	0	0	0	0	0	8	38	44	8
2/8/43	1	5	0	0	2	3	3	57	10	19
9/13/43	1	5	0	0	2	1	2	74	9	6
5/31/44	1	9	0	0	1	2	5	67	12	3

From Sept. 13, 1943, the last date included on the above chart, to May 31, 1944, this patient's blood counts have all remained essentially normal, except for a rise of the leucocyte count to 19,450 in May, 1944. The white count promptly returned to normal after two injections of radioactive phosphorus (1,130 millieuries and 0.518 millieuries, respectively)

Case Summary.—J. M., a 58-year-old married man, first noted slight fatigability sometime during 1940. In January, 1941, the leucocyte count was found to be 88,000 and the diagnosis of myelogenous leukemia was established. He was given a ray irradiation totaling 750 roentgen units, whereupon the white cell count dropped to 12,000. He felt very well and was entirely asymptomatic. However, the leucocyte count gradually rose, and on May 25, 1942, radioactive phosphorus therapy was started because the white cell count had reached 111,000.

Physical Examination (May 25, 1942).—Blood pressure was 155/100. The skin was clean. Lymph nodes were not palpably enlarged. The liver edge was felt 2 cm. below the right costal margin, and the spleen was felt 2 cm. below the left costal margin.

Laboratory Data (May 25, 1942).—Urine was normal. Kahn test was negative. White blood cells, 111,000; red blood cells, 5,510,000; hemoglobin, 15 Gm.; reticulocytes, 2.6 per cent; platelets, 1,664,000; differential: basophiles, 9 per cent; "B" myelocytes, 2 per cent; "C" myelocytes, 22 per cent; juveniles, 22 per cent; stabs, 13 per cent; segmented neutrophils, 26 per cent; lymphocytes, 2 per cent; monocytes, 4 per cent.

A bone marrow examination done a year previously (May 15, 1941) showed a hyperplastic marrow in which 51 per cent of the cells were myelocytes. Very few nucleated red blood cells were seen.

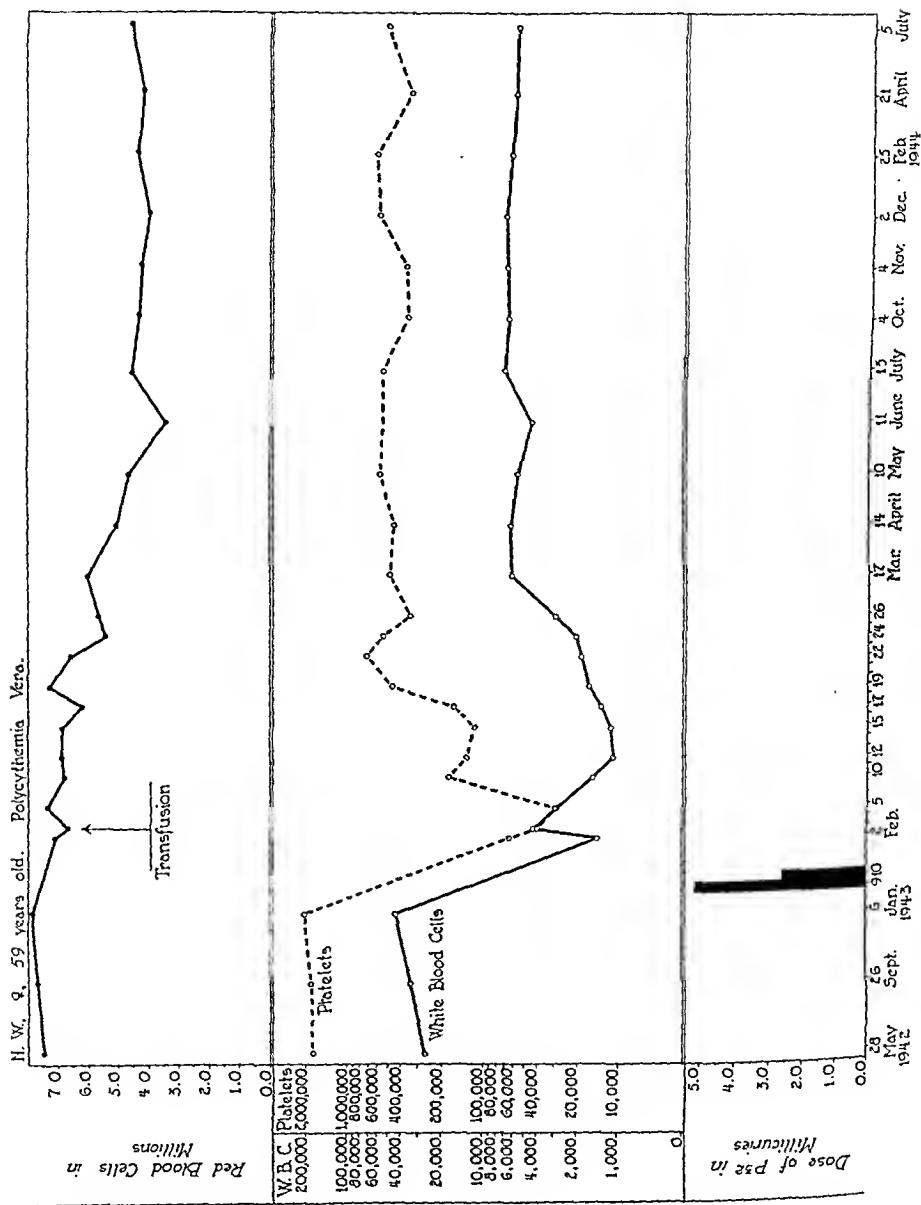


Fig. 2.—For legend see opposite page.

FIG. 2

THERAPEUTICALLY INDUCED LEUCOPENIA AND THROMBOCYTOPENIA IN A PATIENT WITH POLYCYTHEMIA VERA

Case Summary.—N. W., a 59-year-old married woman, had always enjoyed good health until the summer of 1940 when she began to tire easily. At about the same time she noted that the fingers were purplish red in color. Weakness increased and gradually the skin of the entire body became bluish red. In the spring of 1941 she became aware of a mass in the right upper quadrant of the abdomen; there was an occasional aching pain in this region. During the following year she developed sharp pains in her finger tips, aching of her legs, pounding headaches, vertigo and tinnitus, dyspnea on exertion, depression, forgetfulness, nosebleeds, and visual disturbances.

The patient was admitted to Barnes Hospital in February, 1942. The erythrocyte count at that time was 10,535,000 per cubic millimeter and the hemoglobin was 22.5 Gm. Treatment consisted of repeated venesections; a total of 4,200 c.c. of blood was removed. At the time of discharge from the hospital the erythrocyte level was 5,800,000 per cubic millimeter, and the hemoglobin was 14.7 Gm. Symptomatically, she was considerably improved.

For the following year she was treated by periodic venesection, averaging one pint of blood withdrawn every eight weeks. After each phlebotomy she felt weak for a day, following which she would be relatively asymptomatic for from four to six weeks. There would then be a gradual return of all her symptoms.

Radioactive phosphorus therapy was given Jan. 9 and 10, 1943.

Physical Examination (Jan. 9, 1943).—Blood pressure was 170/110. The finger tips, lips, ears, tongue, and pharynx were purplish red. There were numerous small petechiae over the shoulders and lower extremities and a large bruise on the left forearm. Lymph nodes were not palpable. The heart was moderately enlarged, and a systolic murmur was heard at both the apex and base. The lungs were clear. A firm, nontender spleen was felt extending 4 cm. below the left costal margin; the liver edge was felt 5 cm. below the right costal margin.

Laboratory Data.—Urine was normal. Kahn test was negative. Basal metabolic rate, +26 per cent. Blood counts (Jan. 6, 1943).—red blood cells, 7,830,000; hemoglobin, 12.5 Gm.; white blood cells, 43,700; reticulocytes, 1.6 per cent; platelets, 2,040,000. The differential was normal. The bone marrow was examined but not until a week after radioactive phosphorus therapy had been given, the marrow was hyperplastic but the differential was not unusual except for the presence of large numbers of primitive cells.

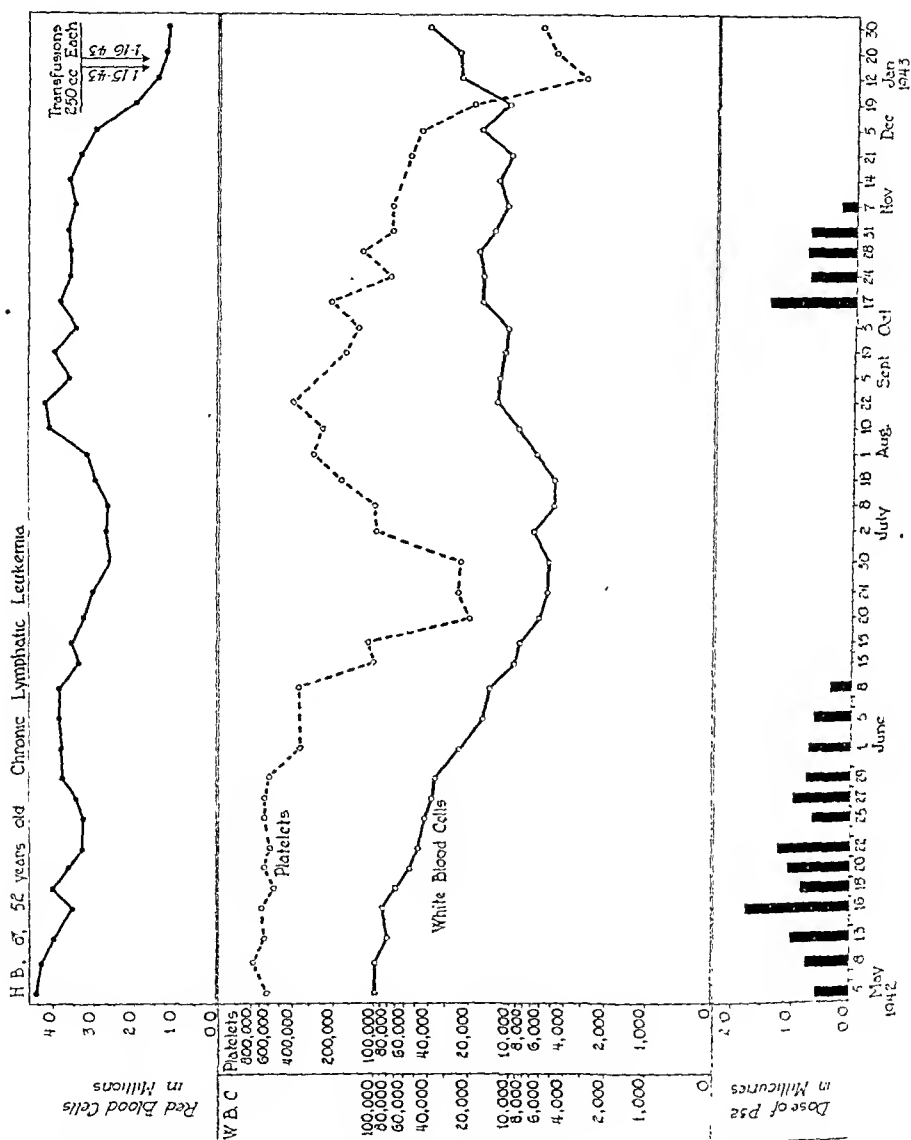


Fig. 3.—For legend see opposite page.

FIG. 3

THROMBOCYTOPENIA OCCURRING IN A PATIENT WITH CHRONIC LYMPHATIC LEUKEMIA FOLLOWING THERAPY WITH P₃₂

Case Summary.—H. B., a 52-year-old married man, first noticed a lump in the left side of the neck in January, 1938. Shortly thereafter the blood was examined and he was told he had leukemia. He was treated with capsules (contents unknown). Very gradually over a period of several years other glands became enlarged.

There were no constitutional symptoms until October, 1941, when the nodes became so large as to make it difficult for him to swallow or turn his neck. He felt weak and tired, and his gums bled whenever he brushed his teeth. He was given ten x-ray treatments totaling 1,000 roentgen units. There was transient symptomatic improvement with decrease in the size of his glands, but six weeks later his glands were larger than before. In January, 1942, he was given x-ray therapy again (nine treatments, totaling 790 roentgen units). This time improvement lasted only about a month. From February to May, 1942, the glands increased in size, he developed pain in his back, and *his urine became steadily weaker*. Treatment with radioactive phosphorus was started May 5, 1942.

Physical examination at that time revealed enlarged lymph nodes in the neck, axillae, and inguinal regions. Some of these were large enough to have a diameter of 4 cm. Even enlarged thymic glands were easily felt. There was slight edema of the face and neck. The edge of the spleen was felt 5 cm. below the costal margin. There were no other significant findings.

Laboratory Data (May 5, 1942).—White blood cells, 98,500; red blood cells, 4,410,000; hemoglobin, 12.8 gm.; reticulocytes, 0.8 per cent; platelets, 620,000; differential: myelocytes, 1 per cent; segmented neutrophils, 8 per cent; lymphocytes, 89 per cent; monocytes, 1 per cent. A lymph node was biopsied Aug. 8, 1942; the microscopic appearance of this gland was compatible with the diagnosis of lymphatic leukemia.

The patient died June 12, 1943, during the last four and one-half months of life, he was a patient at another hospital. The erythrocyte and thrombocyte levels remained very low and the leucocyte level fluctuated widely.

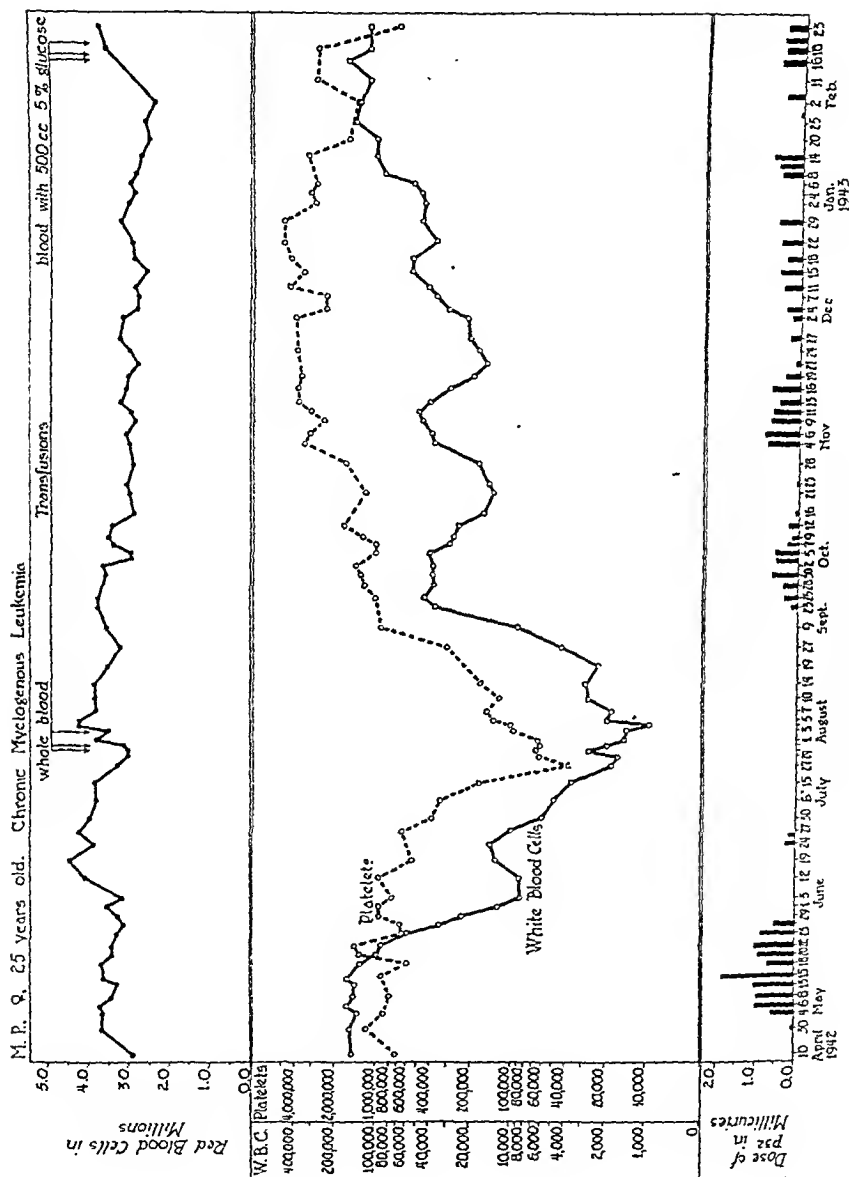


Fig. 4.—For legend see opposite page.

FIG. 4

Case Summary.—M. P., a 25-year-old married woman, developed an itchy, scaly eruption on both eyebrows in March, 1939. This eruption spread down over the lids, and finally over and behind the ears. X-ray treatments and salves were without benefit.

In June, 1939, she had a miscarriage and routine blood counts at this time were: white blood cells, 14,000; red blood cells, 4,470,000; hemoglobin, 87 per cent; differential: segmented neutrophils, 66 per cent; stabs, 7 per cent; lymphocytes 27 per cent. She was again hospitalized in June, 1941, when she delivered a normal infant. Blood counts at this time were: white blood cells, 28,100; red blood cells, 4,230,000; hemoglobin, 81 per cent; differential: segmented neutrophils, 84 per cent; stabs, 7 per cent; lymphocytes, 9 per cent.

Aside from the eruption, which persisted, the patient had no symptoms until January, 1942, when she developed weakness and fatigue. These symptoms persisted until April 30, 1942, when treatment with radioactive phosphorus was started.

Physical Examination (April 30, 1942).—There was a symmetrical skin eruption involving the eyelids, brows, ears, and adjacent areas, the lesion was slightly pigmented, thickened, dry, and scaly, with slightly raised borders. The lymph nodes were not palpably enlarged. The liver and spleen were not palpable (both liver and spleen edges were palpated 2 cm. below the costal margin on May 30, 1942). There were no other significant findings.

Laboratory Data.—Urine was normal. Kahn test was negative. Blood counts (April 30, 1942): white blood counts, 155,000, red blood cells, 3,690,000; hemoglobin, 10.8 Gm.; reticulocytes, 3 per cent; platelets, 1,180,000; differential: basophils, 7 per cent; eosinophiles, 2 per cent; myeloblasts, 2 per cent; "B," myelocytes, 6%; "C," myelocytes, 25 per cent, juveniles, 18 per cent. stabs, 19 per cent; segmented neutrophils, 17 per cent; lymphocytes, 1 per cent; monocytes, 3 per cent. A bone marrow examination was not done until Aug. 18, 1942, 106 days after treatment was started. The bone marrow at this time showed myeloid hyperplasia (52 per cent myelocytes) and only 14 nucleated red blood cells per 100 white blood cells.

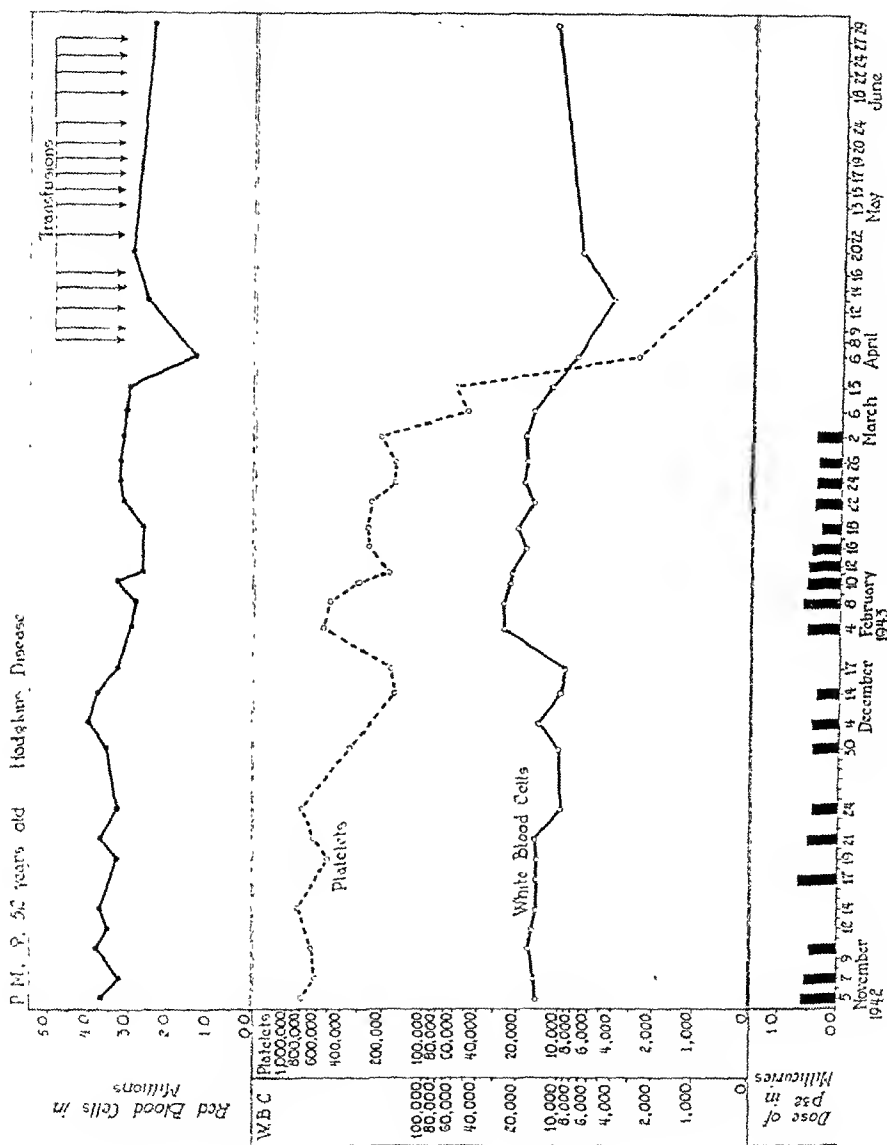


Fig. 5.—For legend see opposite page.

Fig. 5

THROMBOCYTOPENIA FOLLOWING TREATMENT WITH P₃₂ IN A PATIENT WITH HODGKIN'S DISEASE

Case Summary.—P. M., a 52-year-old married woman, enjoyed excellent health until August, 1941, when she first noted a dry hacking cough. In March, 1942, the cough became productive of mucoid sputum. About this same time she began to have dull, aching pain in the left lower chest posteriorly. Shortly thereafter she began to have severe night sweats and increasing fatigue. She lost 12 pounds in weight. An x-ray of the chest in June, 1942, showed extensive pulmonary infiltration. Bronchoscopy revealed only chronic inflammation of the bronchi. By August, 1942, enlargement of the axillary and inguinal lymph nodes was apparent; a biopsy (right axillary node) revealed changes typical of Hodgkin's disease. She was given nine x-ray treatments to the right chest and axilla, following which the pain and cough disappeared. She gained nine pounds. During October the nodes in the left groin increased steadily in size and became quite sore and tender. Radioactive phosphorus therapy was started Nov 5, 1942.

Physical Examination (Nov. 5, 1942).—There was x-ray pigmentation over the right chest and back. A large matted mass of lymph nodes measuring 4 by 3 by 3 cm. was felt in the left inguinal region. The heart and lungs were normal. A firm mass thought to be spleen was felt in the left upper quadrant of the abdomen, but a distinct edge could not be outlined.

Laboratory Data.—Urine was negative except for a faint tract of albumin. Kahn test was negative. Blood counts (Nov. 5, 1942): white blood cells, 14,700; red blood cells, 3,690,000; hemoglobin, 10.1 (gm.); reticulocytes, 2.8 per cent; platelets, 750,000; differential: eosinophiles, 25 per cent; juveniles, 1 per cent; stab, 4 per cent, segmented polymorphonuclear neutrophils, 48 per cent, lymphocytes, 16 per cent, and monocytes, 6 per cent. Sternal bone marrow examination showed 43 per cent eosinophiles, 39 per cent lymphocytes, 6 per cent plasma cells, and only 12 per cent cells of the myeloid series. Very few nucleated red blood cells were seen. There were many globules of fat present.

An x-ray of the chest showed considerable increase in the amount of infiltration in each lung.

tensive purpura on the lower extremities. The platelet count had fallen from 2,000,000 per cubic millimeter to a level of 29,000. Data illustrating three additional instances of the same change are summarized in Figs. 3 to 5.

Of the 44 patients whose platelet levels dropped to less than 100,000 per cubic millimeter, 38 showed clinical manifestations of abnormal bleeding: 22 developed petechiae, purpura, or ecchymoses; 12 bled from the gums; 10 had epistaxis; 6 had hematemesis, hemoptysis, or bloody stools; and 2 had hematuria. Twelve subjects, not included in the foregoing figures, had a thrombocytopenia before treatment was started. Most of these latter patients had acute leukemia, and all had bleeding manifestations. In no case was it felt that bleeding associated with a thrombocytopenia was the principal cause of death, but in several patients in whom the thrombocytopenia was persistent, the bleeding was profuse and may have contributed to the fatal outcome.

It should be emphasized that in many cases radioactive phosphorus therapy was followed by a significant rise in the platelet level. The cases in which the platelet count rose from definitely thrombocytopenic levels to normal values are summarized in Table II.

Many other patients who did not have an initial thrombocytopenia developed a marked increase in the platelet level after they were treated. In 23 of our 100 patients the platelet count increased by 500,000 or more per cubic millimeter at some time during therapy or shortly after treatment was discontinued.

B. LEUCOPENIA.—Following the institution of radioactive phosphorus therapy, the leucocyte levels followed various patterns. In 56 patients there was a steady, progressive decrease in the leucocyte count, in 25 the white blood cell level first increased and then showed a progressive decrease; in 2 patients with acute leukemia there was a gradual rise in the number of leucocytes up to the time of death. Three patients who were in the terminal stages of leukemia first showed a decrease followed by a progressive increase. In the other 14 there was either no significant change in the leucocyte count or a pre-existing trend was apparently uninfluenced by therapy. Thus treatment was attended by an ultimate decrease in the white blood cell level in 81 of the patients, no significant change in 14, and an ultimate increase in 5 with acute or terminal leukemia. The changes in these last 5 patients were probably manifestations of leukemia; it is impossible to tell whether the radioactivity influenced them in any way.

Further analysis of the 18 cases in which the leucocyte level first increased and later progressively decreased is of interest. When treatment was started, the routine followed was to obtain counts every one to four days for the first two weeks, after which the interval was lengthened to once a week. If daily blood counts had been obtained, more patients might have fallen in this group. The interval between the first injection of radioactive phosphorus and the highest peak of the leucocyte curve varied from one to nine days, with an average of four days. The average increase was 17,000 cells with minimum and maximum values of 1,600 and 86,000, respectively; the pretherapeutic basal values had been fairly constant in each case. These observations have added interest in view of the observations of Isaacs and many others, that x-ray therapy is usually followed by a rise in the leucocyte count before the progressive fall sets in.²³

TABLE I

THE INCIDENCE WITH WHICH ANEMIA, LEUCOPENIA, AND THROMBOCYTOPENIA OCCURRED IN 100 PATIENTS AS A COMPLICATION OF THERAPY WITH RADIOACTIVE PHOSPHORUS

	TOTAL CASES	ERYTHROCYTES		LEUCOCYTES		PLATELETS		
		DECREASED 1,000,000 OR MORE (TO < 3.5 M PER C.M.M.)	INCREASED 1,000,000 OR MORE PER C.M.M.	LEUCOPENIA (FAIL TO LESS THAN 3,000 PER C.M.M.)	INITIAL COUNT < 2000 PER C.M.M. TO > 6,000 PER C.M.M.	THROMBOCYTOPENIA		INCREASED BY MORE THAN 500,000 PER C.M.M.
						FALL TO < 100,000 BUT > 50,000 PER C.M.M.	FALL TO < 50,000 PER C.M.M.	
Polycythemia vera	18	4	0	9	0	2	1	3
Myelogenous leukemia	25	8	10	7	0	1	10	13
Lymphatic leukemia	22	7	0	5	0	6	6	12
Leucosarcoma	13	9	1	8	2	1	9	12
Monocytic leukemia	4	0	0	0	0	0	0	0
Plasma cell myeloma	6	12	0	4	0	0	3	1
Lymphosarcoma	12	0	0	1	0	0	0	1
Reticulum cell sarcoma	3	12	0	3	0	0	2	0
Hodgkin's disease	6	12	0	3	0	0	2	0
Giant follicular lymphoblastoma	1	1	0	1	0	0	0	1
Total	100	36	11	41	2	10	33	23

TABLE II

PATIENTS IN WHOM A RISE OCCURRED IN THE PLATELET COUNT FROM THROMBOCYTOPENIC TO NORMAL LEVELS DURING THERAPY WITH RADIOACTIVE PHOSPHORUS

NAME	DIAGNOSIS	PLATELET COUNT AT ONSET OF THERAPY PER C.M.M.	MAXIMUM PLATELET COUNT DURING THERAPY PER C.M.M.
R. G.	Chronic lymphatic leukemia	77,000	347,000
J. S.	Leucosarcoma	16,000	264,000
P. S.	Leucosarcoma	62,000	498,000
D. B.	Monocytic leukemia	72,000	280,000

As shown in Table I, 41 of the 100 patients developed a leucopenia of 3,000 cells per cubic millimeter or less either during or within two months after the cessation of therapy. Here again, those patients who developed a leucopenia which was obviously due to progression of the blood dyscrasia were excluded. The figure of 41 per cent cannot be considered as absolutely accurate, as in some instances it was extremely difficult to determine with certainty whether the therapy or the disease was responsible for the depression of the leucocyte count.

A very severe neutropenia was observed in M. P., a 25-year-old female with chronic myelogenous leukemia. She was started on radioactive phosphorus therapy April 30, 1942, at which time the leucocyte count was 155,000 per cubic millimeter (Fig. 4). Within a period of thirty days she was given intravenously 9.537 millicuries of radioactive phosphorus. On the day of the last injection the white blood cell count was 23,400. Two weeks later, at a time when the leucocyte count was 14,400, she was given two additional minute doses of 0.241 and 0.160 millicuries, respectively. Thirty-six days after the last injection the white blood cell count had fallen to 950 per cubic millimeter,

of which only 9 per cent were polymorphonuclear neutrophils. The only symptoms associated with this agranulocytosis were soreness of the left lower jaw and aching in and below the mandible. The gums appeared dirty and there was a white membrane covering the entire gingival margin. The leucocyte count gradually rose during the subsequent three weeks and the symptoms subsided.

Of the forty-one patients who developed leucopenia apparently as the result of therapy, six had definite ulceration of the mouth or nasopharynx; the other thirty-five had no specific symptoms which could be attributed directly to the leucopenia. Five of these six patients had leucosarcoma, and all had a profound neutropenia at the time therapy with radioactive phosphorus was instituted. The leucopenia which developed rapidly and dramatically in every case represented primarily a destruction of abnormal leukemic lymphocytic cells. Thus, while the rapid decrease in the leucocyte count was unquestionably due to the therapy, the ulceration of mucous membranes might well have occurred even if no treatment had been given.

The sixth patient, however, had polycythemia vera, and here both the rapidly developing leucopenia and the stomatitis were unquestionably due entirely to the therapy. This patient also developed a severe thrombocytopenia (Fig. 2). The leucocyte count prior to treatment was 43,700 cells per cubic millimeter. Twenty-four days after therapy (the equivalent of 5.67 millicuries given intravenously within a period of twenty-four hours) the white blood cell count had dropped to 1,450. In addition to the purpuric manifestations mentioned before, the patient complained of marked weakness and a severe headache. She was hospitalized and was given a blood transfusion in spite of the fact that the erythrocyte count was 6,650,000. The leucocyte count fluctuated between 1,100 and 1,900 for a period of three weeks, after which it gradually rose to normal. During this time she developed an angular-like ulceration on the lower lip. This ulcer persisted for three weeks before finally healing.

C. Anemia.—In Table I it is seen that 36 of the 100 patients developed an anemia which seemed to be due to the therapy. This figure includes only those patients whose erythrocyte level dropped by one million or more cells per cubic millimeter and to a level below 3,500,000. For example, all the patients with polycythemia showed a decrease in erythrocyte count of over 1,000,000 per cubic millimeter, but only the four patients in whom the red blood cell level fell to 3,500,000 cells per cubic millimeter or less are included in the 36 per cent.

In all of the patients the fall in the erythrocyte count was gradual and usually was not manifest for several months after treatment was begun. In only a very few instances was the resultant anemia severe, but the depression of the erythrocyte count was surprisingly persistent. One patient with polycythemia vera still has an anemia eighteen months after the last injection of radioactive phosphorus.

The development of a profound anemia in a patient with polycythemia vera who was treated with moderate doses of radioactive phosphorus therapy is illustrated in Table III. It is interesting that this patient felt better at the time of her most severe anemia than she had prior to therapy, even though she also had a leucopenia and a mild thrombocytopenia. Her only symptoms at present

TABLE III

ANEMIA, LEUCOPENIA, AND MILD THROMBOCYTOPENIA IN A PATIENT WITH POLYCYTHEMIA
VERA FOLLOWING THERAPY WITH RADIOACTIVE PHOSPHORUS

DATE	RADIOACTIVE PHOSPHORUS (MILLICURIES)	R.B.C. PER C.MM.	Hb. (GM.)	WBC PER C.MM.	PLATELETS PER C.MM.
7/14/43		8,110,000	22.3	11,000	1,233,000
7/16/43	0.979				
7/20/43	0.792	7,860,000	22.6	12,150	1,022,000
7/23/43	0.630	7,750,000	21.2	8,300	1,860,000
7/30/43	1.035	7,540,000	21.7	7,450	1,644,000
8/ 4/43	1.148	7,270,000	21.2	4,950	567,000
8/13/43	1.080	7,460,000	22.3	7,550	328,000
9/23/43		5,250,000	13.9	2,850	315,000
12/ 1/43		2,970,000	9.2	2,350	125,000
2/23/44		3,820,000	11.1	3,100	193,000
5/12/44		3,930,000	12.5	4,750	326,000

Total treatment, 5.664.

Case Summary.—R. S., a widowed female 65 years of age, first noted numbness and stiffness of her hands in the winter of 1939-1940. In January, 1941, she began to have pain in the left upper quadrant of the abdomen; she was told by a physician that this was due to an enlarged spleen. The sharp pain disappeared in a few weeks and she has had only occasional aching since. In the spring of 1942 she began to tire easily, she found it necessary to take a nap every afternoon whereas previously she had never slept during the day. She began to lose weight. In May, 1943, she noticed numbness of the feet at night. Occasionally she would wake up screaming with severe pain in her toes. She had headaches about once a week, never very severe.

Physical examination at the time treatment with radioactive phosphorus was started (July 16, 1943) revealed the following: Blood pressure was 185/115. Skin and mucous membranes were deep red in color with some cyanosis of lips, ears, and finger tips. Hair dyed (henna). The neck was stiff and there was limitation of movement in all directions as well as pain in the cervical spine. The heart was enlarged. A hard, nontender spleen was felt extending 18 cm. below the left costal margin. A sharp liver edge descended 4 cm. below the right costal margin on inspiration. There were no other abnormal findings. Weight was 115 pounds and height was 62 inches.

Laboratory Data.—For cytologic studies of peripheral blood (see Table III). Sternal aspiration of bone marrow (July 13, 1943) produced clumps of marrow which were hard and failed to spread under the weight of the cover slip in the usual manner. Microscopically, the marrow was very hyperplastic; erythroid cells as well as myeloid elements and megakaryocytes were present in increased numbers. Blood volume as determined by the method of Gibson and Evans, modified to permit use of the Evelyn photoelectric colorimeter, showed the plasma volume to be 2,380 c.c. and the total blood volume, 7,780 c.c. Blood chemistry studies were all normal. The basal metabolic rate was +15 per cent. Electrocardiogram showed right bundle branch block and occasional auricular premature contractions. The lungs were clear on fluoroscopic examination.

are referable to severe hypertrophic osteoarthritis of the spine and arterio-sclerotic heart disease (electrocardiograms show a right bundle branch block). These changes were induced by a total dose of only 5.66 millieuries of radioactive phosphorus. From the data recorded in Table IV, furthermore, it can be seen that there was a marked difference in the susceptibility of the eighteen patients with polycythemia to the toxic effects of radiophosphorus. Some subjects tolerated comparatively large doses, while others developed thrombocytopenia, leucopenia, and/or anemia when given much smaller amounts.

TABLE IV
HEMATOLOGIC DATA IN EIGHTEEN PATIENTS WITH POLYCYTHEMIA VERA TREATED WITH
RADIOACTIVE PHOSPHORUS

PATIENT	RADIOACTIVE PHOS- PHORUS THERAPY (MILLI- CURIES)	DURATION OF COURSE OF THERAPY (DAYS)	TIME FROM FIRST TREATMENT TO INITIAL DROP IN R.B.C. LEVEL (DAYS)	R.B.C. COUNT AT TIME THERAPY WAS BEGUN (M. PER C.M.M.)	LOWEST R.B.C. COUNT	TIME FROM LOWEST R.B.C. COUNT UNTIL R.B.C. LEVEL WAS AGAIN TO 4,000,000 OR ABOVE (DAYS)	PLATELET COUNT AT TIME THERAPY WAS BEGUN (PER C.M.M.)	LOWEST PLATELET COUNT AFTER THERAPY (PER C.M.M.)	W.B.C. COUNT AT TIME THERAPY WAS BEGUN (PER C.M.M.)	LOWEST W.B.C. COUNT AFTER THERAPY (PER C.M.M.)
D. F.	7.63	34	40	8.59	3.29	163	2,490,000	358,000	15,450	3,400
I. M. C.	5.24†	69	40	7.33	4.77	28	264,000	230,000	18,750	4,200
C. R.	5.63	189	34	6.66	5.47	--	3,685,000	258,000	21,600	3,950
N. W.	7.41	112	30	6.56*	4.59	--	2,036,000	29,000	43,700	1,100
O. A.	9.50§	2	30	7.83	3.35	31	756,000	191,000	5,400	2,500
W. L.	5.74	66	88	9.00	3.19	286	458,000	454,000	4,600	3,200
P. R.	8.44	70	41	6.65	4.75	†	2,080,000	287,000	13,500	1,500
R. S.	13.25	75	33	7.00*	3.37	138	1,233,000	107,000	11,100	1,200
F. V.	5.66	29	40	8.11	2.97	163+	1,266,000	626,000	15,750	7,050
W. W.	6.71	92	60	7.91	4.77	--	2,016,000	250,000	5,000	1,650
K. H.	10.5	49	61	7.15*	3.27	182+	1,818,000	164,000	11,600	4,100
M. T.	4.90	43	28	7.91*	4.34	--	2,600,000	744,000	12,550	5,450
S. K.	7.78	139	30	7.56	4.99	--	3,300,000	476,000	17,200	7,150
O. W.	6.92	120	47	6.47*	5.60	--	820,000	364,000	9,900	4,300
A. B.	5.91	87	60	8.01	5.12	--	1,582,000	153,000	9,650	950
W. M.	5.17	2	48	7.57	3.50	30+	1,607,000	191,000	10,400	1,550
M. K.	4.07	1	58	7.98	4.47	--	1,250,000	260,000	12,000	1,150
W. S.	4.03	1	41	6.02*	4.46	--	1,543,000	724,000	13,300	5,250
W. S.	4.14	44	83	5.78*	3.54	10+				

*Patient treated by phlebotomies or phenylhydrazine up to the time ^{32}P therapy was begun. It is probable that there was greater erythroid activity than was suggested by the red blood cell count.

†Patient not seen since July, 1943, at which time the red blood count was 4.87 million. Lowest count, 4.75 million, was in June, 1943.

‡First course of treatment (shown above) brought the red blood count down to 4.77 million. Three months after the last injection in this series of treatments, the red blood count had risen to 6.66 million and the patient was beginning to have headaches again. Second course of treatment (shown below) was therefore given.

§12.665 milllicuries given orally which is equivalent to 9.50 milllicuries I.V. (assuming 75 per cent absorption from the gastrointestinal tract).

Most of the patients who developed a significant anemia complained of weakness and fatigability. However, in many cases these symptoms were present prior to therapy, and it was not possible to ascertain the percentage of patients who developed symptoms as a direct result of the anemia.

D. Aplasia of the Bone Marrow.—At necropsy, localized areas of necrosis in the bone marrow were observed several times. In four instances, the marrow was found to be aplastic. This change occurred in the tissues of J. S., a boy 14 years of age who had leucosarcoma. When first seen, he had 4,400,000 red blood cells per cubic millimeter, 13.7 Gm. of hemoglobin, 15,600 leucocytes, and a platelet count of less than 10,000. Ninety-four per cent of the white blood cells were abnormal, immature lymphocytes. Bone marrow obtained by sternal aspiration showed an almost solid mass of leukemic cells; granulocytes and erythroid elements were rare; no megakaryocytes were seen. In the hope that therapy might lead to destruction of enough leukemia cells to permit formation of normal blood cells by the bone marrow, 6,858 millicuries of radioactive phosphorus were given over a period of twenty-eight days. Although the platelets remained low, they did rise temporarily to 44,000 per cubic millimeter. The white blood cell count, however, fell to between 100 and 500 cells, and the number of erythrocytes decreased steadily to 1,670,000 cells at the time of death, only thirty-one days after institution of therapy. The marrow at necropsy showed many areas of hemorrhage in addition to the aplasia. There were practically no morphologic evidences of leukemia in any of the tissues. Two of the other patients who developed aplasia of the marrow also had leucosarcoma, while the fourth had Hodgkin's disease. X-ray therapy had also been given in this latter instance. The four cases are similar to those studied by Kenney¹⁶ and by Diamond and Warren.¹⁷ These observations, coupled with those which demonstrate that aplastic anemia may be produced in animals to which large doses of radioactive phosphorus are given,¹⁸ emphasize the fact that caution must be exercised if the production of irreversible toxic effects on the marrow are to be avoided.

DISCUSSION

Description of these hematologic complications of radioactive phosphorus therapy is in no way intended to deprecate the therapeutic value of radio-phosphorus. In almost all instances there was prompt recovery from the thrombocytopenias and leucopenias, with slow recovery from the anemias which had apparently been induced by the isotope. The only exceptions occurred among patients who were obviously in the terminal stages of their disease. The experience being accumulated in this clinic confirms the conclusions of other workers, that radioactive phosphorus induces excellent remissions in patients with polycythemia vera, chronic myelogenous leukemia, and chronic lymphatic leukemia. The results are at least as good as those obtained with other forms of radiation therapy; they may prove to be better. Since, however, it is important to understand the dangers as well as the limitations of any new therapeutic technique, the occurrence of these complications should receive the emphasis given to them in this report. This is particularly true because if they are recognized and further therapy is withheld, the production of irreversible damage to the hematopoietic organs may be avoided.

The higher incidence of thrombocytopenia, leucopenia, and anemia in this series of cases than in those reported from other laboratories may, in part, be explained by the fact that an attempt was made to bring the blood count of patients with leukemia to normal levels. This was done to obtain the maximum therapeutic effectiveness of the radiant energy. Most other investigators have stopped short of this goal and have preferred, rather, to treat their patients symptomatically.

As is emphasized in Table IV, however, there is a wide variation in the individual susceptibility to radioactive phosphorus therapy. The patients with polycythemia vera received doses of the material which were comparable to those used in other clinics.^{10, 11} Some of the more severe complications were shown by subjects who had been given relatively small amounts of radiophosphorus, while others who were given large amounts tolerated them well. Because of the variation in susceptibility to the effects of this form of radiation therapy, dosage regulation must be individualized to a high degree. The hematologic complications may be so delayed, however, that even when great caution is exercised, they may appear weeks or months after the last dose of radioactive phosphorus has been given.

Emphasis should also be given to the fact that therapeutically induced remissions were attended by a rise in the red blood cell count of over a million cells in eleven instances and by a rise of more than 500,000 platelets per cubic millimeter in twenty-three. Two patients with subleukemic leukemia and an initial white cell count of less than 2,000 showed increases in the leucocytes to more than 6,000 (Table I). These effects are similar to those often seen after x-ray therapy.

SUMMARY AND CONCLUSIONS

1. Of 100 patients treated with radioactive phosphorus for various hematologic dyscrasias, 44 developed a thrombocytopenia of less than 100,000 per cubic millimeter, and in 33 of these it was less than 50,000; 41 showed a leucopenia of less than 3,000; and in 36 the red blood cells fell by more than 1 million cells to a level of under 3.5 million. When more than one of these changes occurred in the same individual, the white blood cells usually decreased first, the platelets second, and the red cells third. Thrombocytopenia and anemia frequently appeared as late manifestations, occurring even weeks after therapy had been discontinued.

2. Thirty-eight of the patients with thrombocytopenia appearing after therapy showed clinical manifestations of abnormal bleeding, and in one patient with polycythemia with severe neutropenia there developed an angular-like lesion of the lower lip. Symptoms of weakness and fatigue appeared in several of the subjects whose red cell count fell to anemic levels.

3. An aplasia of the marrow was apparently induced by therapy with radioactive phosphorus in three cases of leucosarcoma and one of Hodgkin's disease.

4. As was best demonstrated in the patients with polycythemia vera, there was considerable variation in the susceptibility of different subjects to the toxic effects of radioactive phosphorus. In several instances, leucopenia, thrombocytopenia, or an anemia followed comparatively small doses of the material.

Much larger amounts were tolerated by other patients without difficulty. Dosage must be individualized to a high degree.

5. While the conclusion of other investigators, that radioactive phosphorus is a valuable therapeutic agent in the treatment of polycythemia and chronic leukemias, is confirmed, emphasis is given to the observation that severe thrombocytopenia, leucopenia, a moderate anemia, and aplasia of the bone marrow may occur as complications of the therapy. The blood of patients treated in this manner should be studied at frequent intervals so that the hematologic changes can be recognized early and further administration stopped before irreversible toxic effects on the bone marrow are produced.

REFERENCES

1. Lawrence, J. H., Scott, K. G., and Tuttle, L. W.: Studies on Leukemia With the Aid of Radioactive Phosphorus, *Internat. Clin.* 3: 33-58, 1939.
2. Lawrence, J. H.: Nuclear Physics and Therapy: Preliminary Report on a New Method of Treatment of Leukemia and Polycythemia, *Radiology* 35: 51-60, 1940.
3. Warren, S.: The Treatment of Leukemia by Radio-Active Phosphorus, *New Eng. J. Med.* 223: 751-754, 1940.
4. Erf, L. A., and Lawrence, J. H.: Clinical Studies With the Aid of Radio-Phosphorus: Absorption and Distribution of Radio-Phosphorus in the Blood of, Its Excretion by, and Its Effect on Patients With Polycythemia, *Ann. Int. Med.* 15: 276-290, 1941.
5. Erf, L. A., Tuttle, L. W., and Lawrence, J. H.: Clinical Studies With the Aid of Radio-Phosphorus. Retention in Blood, the Excretion and the Therapeutic Effect of Radio-Phosphorus on Patients With Leukemia, *Ann. Int. Med.* 15: 487-543, 1941.
6. Lawrence, J. H.: Observations on the Nature and Treatment of Leukemia and Allied Diseases, *Proc. Inst. Med. Chicago* 14: 30-49, 1942.
7. Keuney, J. M.: Radioactive Phosphorus as a Therapeutic Agent in Malignant Neoplastic Disease, *Cancer Research* 2: 130-145, 1942.
8. Craver, L. F.: Treatment of Leukemia by Radioactive Phosphorus, *Bull. New York Acad. Med.* 18: 254-262, 1942.
9. Fitz-Hugh, T., Jr., and Hodes, P. J.: Clinical Experience With Radio-Phosphorus in the Treatment of Certain Blood Dyscrasias, *Am. J. M. Sc.* 204: 662-665, 1942.
10. Low-Beer, B. V. A., Lawrence, J. H., and Stone, R. S.: The Therapeutic Use of Artificially Produced Radioactive Substances. Radiophosphorus, Radiostrontium, Radiiodine, With Special Reference to Leukemia and Allied Diseases, *Radiology* 39: 573-597, 1942.
11. Erf, L. A., and Jones, H. W.: Radiophosphorus—Agent for Satisfactory Treatment of Polycythemia and Its Associated Manifestations; Case of Polycythemia Secondary Possibly to Banti's Syndrome, *Ann. Int. Med.* 19: 587-601, 1943.
12. Minot, G. R., and Spurling, R. G.: Effect on Blood of Irradiation, Especially Short Wave Length Roentgen-Ray Therapy, *Am. J. M. Sc.* 168: 215, 1924.
13. Robbins, L. L.: Roentgen Irradiation in Polycythemia Vera by Multiple Small Doses to Large Areas of the Body, *Am. J. Roentgenol.* 51: 230-235, 1944.
14. Scott, K. G., and Cook, S. F.: The Effect of Radioactive Phosphorus Upon the Blood of Growing Chicks, *Proc. Nat. Acad. Sc.* 23: 265, 1937.
15. Scott, K. G., and Lawrence, J. H.: Effect of Radio-Phosphorus on Blood of Monkeys, *Proc. Soc. Exper. Biol. & Med.* 48: 155, 1941.
16. Kenney, J. M., and Craver, L. F.: Further Experiences in the Treatment of Lymphosarcoma With Radioactive Phosphorus, *Radiology* 39: 598-607, 1942.
17. Diamond, L. K., and Warren, S.: Personal communication.
18. Wiseman, B. K.: Lymphopoiesis, Lymphatic Hyperplasia and Lymphemia: Fundamental Observations Concerning the Pathologic-Physiology and Inter-Relationship of Lymphatic Leukemia, Leukosarcoma and Lymphosarcoma, *Ann. Int. Med.* 9: 1303, 1936.
19. Kamen, M.: Personal communication.
20. Evelyn, K. A.: A Stabilized Photoelectric Colorimeter With Light Filters, *J. Biol. Chem.* 115: 63, 1936.
21. Dameshek, W.: Method for Simultaneous Enumeration of Blood Platelets and Reticulocytes With Consideration of Normal Blood Platelet Count in Men and in Women, *Arch. Int. Med.* 50: 579, 1932.
22. Hamilton, J. G.: The Use of Radioactive Tracers in Biology and Medicine, *Radiology* 39: 541, 1942.
23. Isaacs, R.: The Relation of Cell Types in Leukemia to Sensitivity to Radiation, *Folia Haematologica* 52: 414, 1934.

SALMONELLA FOOD INFECTION IN MILITARY PERSONNEL

AN OUTBREAK CAUSED BY *S. ORANIENBURG*, *S. TYPHI MURIUM*, AND *S. ANATUM*

CAPTAIN WILLIAM GREIFINGER, M.C., AND JOSEPH K. SILBERSTEIN, M.A.

FOOD infection is defined by Dunham¹ as an acute gastroenteritis caused by the ingestion of food contaminated with specific organisms. The paratyphoid or Salmonella group is the most common offender. Herein is described an outbreak of 115 cases of food infection in military personnel caused by three members of the Salmonella group, specifically *S. oranienburg*, *S. typhi murium*, and *S. anatum*.

CLINICAL OBSERVATIONS

On July 25, 1943, twenty-eight individuals, all males from 21 to 50 years of age, were admitted to the hospital complaining of nausea, vomiting, abdominal cramps and diarrhea of sudden onset. Bowel movements were frequent, eight to fifteen per day, loose, watery, brownish-green in color. Gross or occult blood was present in fourteen of the patients' stools. In ten specimens there was pus, three of which had considerable pus and mucus. No ova or parasites were found in any of the stools examined.

Those patients who had nausea and abdominal cramps were rid of these symptoms in 24 to 48 hours. The diarrhea lasted longer, the range in duration being 1 to 6 days, average 3 days. All patients admitted one to two days after the onset of illness had temperature elevation, 101° F. to 104° F. There was a slight leucocytosis present in the blood counts taken at this time. None of the patients was prostrated and none died.

All patients had been eating at the same mess daily. Epidemiological studies were made by investigators in an attempt to determine the cause of the outbreak. Those common foods eaten by most of the men at the evening mess were fried fish, tartar sauce, and rice pudding. At the time of the onset of symptoms, none of this food was available for laboratory examination. The fish was suspected because it had been noted that the ice refrigerating it had melted. The incubation period appeared to be 6 to 24 hours after the ingestion of the food.

In 72 hours, the first cultural reports of the stools were presumptively positive for Salmonella, and consequently eighty-seven additional men who had eaten at this mess were hospitalized. Most of this latter group had normal stools and the usual number of bowel movements. They gave a history of having had mild diarrhea prior to admission, however. (Table I.) Some who had no symptoms were later found to have positive stool cultures.

It was decided to retain these men in the hospital until 3 consecutive stool examinations were negative for Salmonella. Stools were cultured twice weekly

TABLE I

SYMPTOM	NO. PATIENTS	PER CENT PATIENTS TOTAL 115
Nausea	25	21.7
Vomiting	4	3.5
Cramps	83	72.2
Diarrhea	107	93.0
Shock	0	0
Death	0	0

until the number of patients decreased sufficiently to permit examinations at more frequent intervals.

LABORATORY OBSERVATIONS

Fecal specimens were obtained in paraffin-coated sputum cups. Since the patients were in the hospital, these specimens were readily obtained and no preservative was necessary. In the laboratory they were examined as to color, character, and the presence of gross blood. Where there was no blood visible, the orthotolidine test for occult blood was performed as described by Simmons.² A small portion of the stool was emulsified, using physiological saline solution. This emulsion was placed on a microscope slide and examined for red blood cells, mucus, pus, ova, and parasites, especially *Endamoeba histolytica*.

A systematic method as outlined by Littman³ was found most essential in handling the specimens involved in this outbreak. A chart was made consisting of the patients' names arranged alphabetically, dates, and bacteriological findings. After the inoculation of the culture media, all specimens were incinerated. It was deemed impractical to keep stools for restreaking as recommended by Mayfield and Gober.⁴

For bacteriological examination the following routine was employed. SS (Shigella-Salmonella) plates were heavily streaked. A portion of feces about the size of a bean was placed in selenite-F enrichment broth and both media were incubated 18 to 24 hours. At the end of this time, three to four colorless colonies or those with bull's-eye appearance on SS agar were fished and inoculated into slants of Kligler iron agar. The enrichment culture medium was streaked heavily onto SS agar. Both media were incubated 18 to 24 hours. The Kligler iron agar slants were then observed for fermentation.

Gram's stain and motility tests were made from growth on these slants. At this time, if there was a growth of a gram-negative, motile, nonsporing bacillus which produced acid and gas in the butt, hydrogen sulfide, and an alkaline slant, it was reported as follows, "Presumptive test positive for Salmonella—confirmative report to follow."

It was thought impractical to carry all cases of this outbreak to a conclusion at this point, because of the large number of specimens involved. By the selection of representative cultures from the total number of cases presumptively positive for Salmonella and employing the necessary carbohydrate fermentation and serologic tests, the etiological agents were identified.

In our bacteriological examinations, the following differential media were employed: desoxycholate-citrate agar, B.B.L.,⁵ eosin-methylene blue agar, Difco⁶ and SS agar, Difco.⁶ The medium yielding the largest percentage of positives in our experience was SS agar. This is in accord with the findings of Mayfield

and Gober,⁴ Hardy, Watt, and DeCapito,⁷ Daek⁸ and others. It was found that the plates of this medium could and should be heavily inoculated. They did not produce an overgrowth, however, as was noted with eosin-methylene blue agar plates. They also remained clear whereas the desoxycholate-citrate agar plates became cloudy and opaque, especially when lactose-fermenting organisms were present in any number. The good results obtained with this medium, its simplicity of preparation and economy recommend its use.

An enrichment medium such as Leifson's⁹ selenite-F, B.B.L.⁵ was found to be invaluable in the culturing of specimens from carriers and in those containing few enteric organisms. It is essential, however, to streak the stools initially on a plate as well as inoculating an enrichment medium because *Proteus* or *Pseudomonas* may overgrow the *Salmonella* when either is present. Also, when there are more than one *Salmonella* species present, one species may overgrow the other in this enrichment broth.

As a fermentation test medium, Kligler iron agar⁶ was found to be more satisfactory than Russell double sugar agar because it contains an iron salt which is an excellent indicator of hydrogen sulfide production. The use of this medium in 3 to 4 c.c. amounts, in Wassermann or Loeffler tubes, effected a great saving of material.

Not all the carbohydrates initially employed by us were found to be essential. The use of dextrose, lactose, sucrose, maltose, mannite, inositol, tryptone water (indol), salicin, and D-sorbitol as recommended by Saphra and Silberberg¹⁰ were found to be satisfactory for biochemical study.

Final identification was made serologically using spot group agglutinations, and then the antigenic structure of the *Salmonella* was determined using the Kauffmann-White schema.¹¹ This identification was made in the laboratory of the New York *Salmonella* Center, Beth Israel Hospital, New York.

CLINICAL COURSE AND THERAPY

Patients were treated symptomatically on admission to the hospital and by the end of the first week the symptoms of gastroenteritis had subsided. When the stool cultures revealed the causative organisms to be *Salmonella*, treatment with sulfaguanidine (sulfanilyl guanidine) was initiated. This chemotherapeutic agent was first studied by Marshall and his colleagues,¹² and suggested for the treatment of bacterial intestinal infections. This sulfonamide is slightly water-soluble, and poorly absorbed from the intestines. A course of 16 Gm. of the drug was given to all patients between Aug. 2 and Aug. 5, 1943 (second week). That this was slightly beneficial is shown in Fig. 1, where it is noted that there was a slight decrease in the census of patients confined because of this outbreak. It is to be recalled that no patient was discharged to duty until three consecutive negative stools were obtained. A second course of sulfaguanidine, 4 Gm. per day, was given to the patients from Aug. 21 to Aug. 23 (fourth week). A few patients were found to have three negative stools following this course. It is notable that after this second administration of sulfaguanidine, *S. typhi murium* was not recovered again in any stools. There was no reaction to the drug. Blood levels of sulfaguanidine were under 1 mg. per cent.

A course of sulfasuxidine consisting of 4 Gm. daily for 3 days was given to all patients Sept. 23 to 25 (ninth week). It was noted that there was a reduction of enteric pathogens (nonlactose-fermenting organisms) on differential media. *S. anatum* was decreased markedly (see Fig. 1), while *S. oranienburg* was decreased slightly.

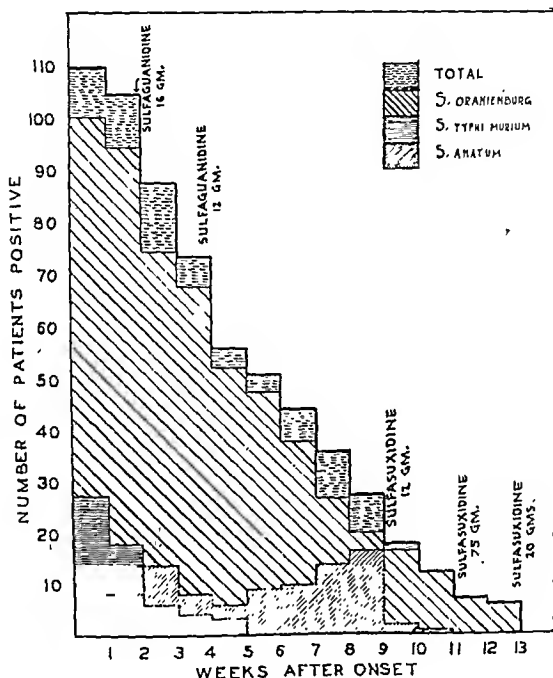


Fig. 1.

Because of the satisfactory results obtained with this dosage, the 12 remaining carriers at the eleventh week were subjected to larger quantities of this sulfonamide. Starting Oct. 13 and for 5 successive days, a total of 75 Gm. was given to 6 of these patients. All these patients' stools were free of *Salmonella* thereafter. The other 6 were used as controls. Two of these became negative spontaneously, while four remained persistently positive for *S. oranienburg*. These carriers were then given 20 Gm. of sulfasuxidine in 2 days and they too became *Salmonella* free, as determined by seven daily negative stools.

There were no toxic reactions following this sulfatherapy. Blood counts taken during this period were within the normal range. Urine analyses revealed no pathological findings. None of the patients complained of nausea, headache,

or rash. The concentration of this sulfonamide in the blood was consistently low, and usually less than 1 mg. per cent. This tends to confirm the findings of other investigators that a high concentration of this drug is maintained in the gastrointestinal tract with a low concentration of the drug in the blood.

PERSISTENCE OF SALMONELLA INFECTION

The incidence and persistence of the enteric pathogens in the 115 cases is shown in Table II. As previously stated, this outbreak was due to a multiple infection of *Salmonella*. The incidence was recorded and presented graphically in Fig. 1 in reference to the different species of *Salmonella* cultured from the patients' stools either during or subsequent to the week stated. Each patient was considered positive as long as one of the *Salmonella* was recovered from his stool.

During or following the first week, 109 (94.8 per cent) patients were found to have stool cultures positive for one or more of the *Salmonella*. *S. oranienburg* was recovered from 100 (86.9 per cent) patients, whereas *S. typhi murium* appeared in 27 (23.4 per cent). In 6 (5.2 per cent) patients the etiological agents were never recovered.

TABLE II

PERSISTENCE OF SALMONELLA INFECTION AS DETERMINED BY FECAL CULTURES IN 115 PATIENTS

WEEK FOLLOWING INGESTION OF SUSPECTED FOOD	NUMBER AND PER CENT PATIENTS POSITIVE						TOTAL NUM- BER POSITIVE CASES SINGLE OR MULTIPLE	PER CENT TO- TAL POSITIVE CASES SINGLE OR MULTIPLE
	<i>S. oranien- burg</i>		<i>S. typhi murium</i>		<i>S. anatum</i>			
	NO.	%	NO.	%	NO.	%		
First	100	86.9	27	23.4	0	0	109	94.8
Second	94	81.7	18	15.6	0	0	104	90.4
Third	74	64.3	6	5.2	14	12.2	87	75.7
Fourth	68	59.1	4	3.4	8	6.9	73	63.5
Fifth	52	45.2	3	2.6	6	5.2	56	48.7
Sixth	48	41.7	0	0	9	7.8	51	44.3
Seventh	38	33.0	0	0	10	8.7	44	38.3
Eighth	27	23.5	0	0	14	12.2	36	31.3
Ninth*	20	17.4	0	0	16	13.9	28	24.3
Tenth	16	13.9	0	0	2	1.7	17	14.8
Eleventh	12	10.4	0	0	1	0.9	12	10.4
Twelfth	7	6.1	0	0	0	0	7	6.1
Thirteenth	6	5.2	0	0	0	0	6	5.2
Fourteenth	0	0	0	0	0	0	0	0

*Sulfasuxidine administered.

Table III indicates the true nature of the multiple infection. In pure culture, there were 45 (39.1 per cent) cases of *S. oranienburg*, but only 4 (3.5 per cent) of *S. typhi murium* and 4 (3.5 per cent) of *S. anatum*.

There were various combinations of *Salmonella* in some cases. In 16 (13.9 per cent), *S. oranienburg* and *S. typhi murium* were found, in 33 (28.7 per cent), *S. oranienburg* and *S. anatum*, and 1 (0.9 per cent) had a combination of *S. typhi murium* and *S. anatum*. There were 6 cases of infection with all three species.

In summing up the incidence of each species it is noted that *S. oranienburg* was found in 100 (86.9 per cent) cases, *S. typhi murium* in 27 (23.5 per cent) and *S. anatum* in 44 (38.2 per cent) either in combination or alone.

TABLE III
INCIDENCE OF SALMONELLA INFECTION SINGLE AND MULTIPLE

ENTERIC PATHOGEN	NUMBER PATIENTS POSITIVE	PER CENT PATIENTS POSITIVE
Single:		
<i>S. oranienburg</i>	43	39.1
<i>S. typhi murium</i>	4	3.5
<i>S. anatum</i>	4	3.5
Multiple:		
<i>S. oranienburg</i>	16	13.9
<i>S. typhi murium</i>		
<i>S. oranienburg</i>	33	28.7
<i>S. anatum</i>		
<i>S. typhi murium</i>	1	0.9
<i>S. anatum</i>		
<i>S. oranienburg</i>	6	5.2
<i>S. typhi murium</i>		
<i>S. anatum</i>		
Negative	6	5.2
Total	115	100.0

SEROLOGIC OBSERVATIONS

It is noteworthy that all these patients had received a course of triple typhoid vaccine (TAB) containing *E. typhosa*, *S. paratyphi* (para A) and *S. schottmuelleri* (para B).

Initial bloods were collected on Aug. 6, 2 weeks after onset (see Table IV), and tube agglutination tests were performed at 56° C. for 2 hours, using homologous cultures of *S. typhi murium*, *S. oranienburg* or both.* *S. anatum* was not included at this time, because this organism had not yet been recovered from the stools (see Table II). All of the 23 patients from whom blood was drawn had symptoms, and their stool cultures were positive for one or both of the Salmonella. Four of the five sera tested with *S. typhi murium* showed no agglutination titer, although this organism was recovered from the stools (Table IV, Nos. 7, 9, 12, 14). The remaining one showed a 2+ titer at 1:200 dilution (No. 23). When the bloods were tested with the homologous antigen *S. oranienburg*, however, 18 showed a definite antibody titer, 1:3200 in some instances (Nos. 11 and 12), and the majority agglutinated at 1:200. A few specimens had lower titers, 1:50 and 1:100. In all instances where *S. oranienburg* was recovered from the stool, the homologous antibody was present in the patient's blood at the end of this 2-week period. In the two cases where *S. oranienburg* and *S. typhi murium* were recovered from the stools (Nos. 12 and 14), the bloods titrated with the homologous antigens showed strong titer for *S. oranienburg*, 1:3200 and 1:800, while they were negative for *S. typhi murium* in all dilutions.

Thus, at the end of the second week, it was possible to confirm the diagnosis of Salmonella infection by means of blood agglutination tests.

The second blood specimens were collected from 28 patients on Aug. 14 and titrated with the apparently predominant organism, *S. oranienburg*. Nine

*These tests were performed by M/Sgt. Robert J. Helmold of the Bacteriology Department of the Second Service Command Laboratory.

TABLE IV
SEROLOGIC DATA
(Aug. 6, 1943)

PATIENT	SALMONELLA RECOVERED IN STOOL AND ANTIGEN USED	BLOOD TITER (2 HR. @ 56° C.)						
		SERUM DILUTIONS						
		1/50	1/100	1/200	1/400	1/800	1/1600	1/3200
1	<i>S. oranienburg</i>	2+	2+	0	0	0	0	0
2	<i>S. oranienburg</i>	3+	3+	0	0	0	0	0
3	<i>S. oranienburg</i>	4+	4+	4+	4+	0	0	0
4	<i>S. oranienburg</i>	4+	4+	2+	0	0	0	0
5	<i>S. oranienburg</i>	4+	4+	4+	4+	4+	0	0
6	<i>S. oranienburg</i>	4+	4+	4+	0	0	0	0
7	<i>S. typhi murium</i>	0	0	0	0	0	0	0
8	<i>S. oranienburg</i>	2+	2+	0	0	0	0	0
9	<i>S. typhi murium</i>	0	0	0	0	0	0	0
10	<i>S. oranienburg</i>	4+	4+	4+	2+	0	0	0
11	<i>S. oranienburg</i>	4+	4+	4+	4+	4+	4+	3+
12	{ <i>S. oranienburg</i>	4+	4+	4+	4+	4+	4+	2+
	{ <i>S. typhi murium</i>	0	0	0	0	0	0	0
13	<i>S. oranienburg</i>	4+	4+	4+	4+	3+	0	0
14	{ <i>S. oranienburg</i>	4+	4+	4+	4+	2+	0	0
	{ <i>S. typhi murium</i>	0	0	0	0	0	0	0
15	<i>S. oranienburg</i>	4+	4+	2+	0	0	0	0
16	<i>S. oranienburg</i>	2+	1+	0	0	0	0	0
17	<i>S. oranienburg</i>	4+	4+	4+	4+	4+	2+	0
18	<i>S. oranienburg</i>	2+	0	0	0	0	0	0
19	<i>S. oranienburg</i>	2+	2+	0	0	0	0	0
20	<i>S. oranienburg</i>	4+	4+	4+	4+	4+	2+	0
21	<i>S. oranienburg</i>	4+	4+	4+	4+	2+	0	0
22	<i>S. oranienburg</i>	4+	4+	4+	4+	2+	0	0
23	<i>S. typhi murium</i>	3+	3+	2+	0	0	0	0

4+—coarse flocculation—clear supernatant fluid

3+—coarse flocculation—slightly turbid fluid

2+—fine flocculation—moderately turbid fluid

1+—very fine flocculation—moderately turbid fluid

0—no flocculation—opalescent fluid

of these specimens were from patients whose sera had been tested previously. It was found that there was a persistence and in some instances an increase in antibody titer. In the remaining nineteen new specimens drawn from patients whose stools were known to be positive for this bacillus, definite titers were obtained, varying from 1:100 to 1:3200, the majority being 1:200.

Number of bloods tested previously and now repeated	9
Persistence of titer	4
Increase	3
Slight decrease	2

The third blood specimens were drawn from 69 patients on Aug. 24 (fourth week). They were now tested with *S. oranienburg*, *S. typhi murium* and *S. anatum*, since one or more of these pathogens had been recovered from the patients' stools. Thirty-two of these patients had had at least one previous agglutination test for *S. oranienburg*, with the following results:

Number of bloods tested previously and now repeated	32
Persistence of titer	14
Increase	3
Decrease	15

Sera from the other 37 patients gave a definite agglutination titer for *S. oranienburg* ranging from 1:50 to 1:1600. Patients from whom *S. anatum* and *S. typhi murium* had been recovered showed little or no titer for these organisms. Two cases in which only *S. typhi murium* was found in the stools showed a positive titer for *S. oranienburg* in the patients' blood. In a few instances *S. typhi murium* antibody was 1:50 or 1:100, which may be attributed in part to the TAB vaccine, since *S. schottmuelleri* has a similar somatic antigenic structure.

On Sept. 25 (two months), a group of 31 blood sera was tested. All were titrated against *S. oranienburg*, because, as previously noted, this was the one organism which produced definite antibody reaction.

Number of bloods previously tested and now repeated	26
Persistence in titer	8
Increase	3
Decrease	15

Eighteen of these sera still had a relatively high titer for this Salmonella, about 1:400 and as high as 1:3200 in some instances. There were 2 cases in which *S. anatum* was the only organism recovered in the stool. Their blood sera, however, showed a high titer, 1:1600 for *S. oranienburg*, again proving the multiplicity of the infection. Sera from the 5 patients tested for the first time gave a definite agglutination titer for *S. oranienburg*.

On Oct. 22, 9 blood sera were examined for antibody titer with *S. oranienburg*. All showed a decrease in titer, although in some it persisted, 1:400.

Number of bloods previously tested and now repeated	9
Persistence in titer	0
Increase	0
Decrease	7
Decrease to negative	2

In conclusion, a rapid titer was produced at the end of the second week for *S. oranienburg*, slight or no titer for *S. typhi murium* and *S. anatum*. There was a gradual decrease and at the end of three months there was but weak antibody present, in some instances none.

DISCUSSION

There have been several reports of multiple enteric infections described in the literature, especially in reference to epidemics. Edwards and Bruner¹³ noted multiple infections caused by various paratyphoid bacilli in fowl. Bornstein,¹⁴ Hormaeche, et al.¹⁵ and Schiff and Saphra¹⁶ described human cases of food infection in which more than one species of Salmonella were recovered in the stools. That this is also the case in outbreaks of dysentery has been stated by Topley and Wilson¹⁷ and by Hardy and Watt.¹⁸ In this outbreak three distinct species were involved, *S. oranienburg*, *S. typhi murium*, and *S. anatum*.

S. oranienburg was found in 100 of the cases, by far the greatest incidence of specific Salmonella. Because of this and the positive blood serum agglutinations obtained with this organism, it may be considered the most important etiological agent. It is evident, however, in this outbreak, that all three Salmonella were originally ingested with the contaminated food.

S. oranienburg was first isolated from the stool of a healthy boy and described by Kauffmann¹⁹ in 1930. The Kauffmann-White schema¹¹ lists this organism in group C as having the following antigenic structure: VI₁.VI₂.VII: m,t, :—.

In 1936, Edwards²⁰ described a case of *S. oranienburg* in quail. Schiff and Strauss,²¹ in 1939, identified this organism in the feces of a three-year-old child with gastroenteritis, the first such case to be recorded in the United States.

S. anatum was first isolated from ducklings by Rettger and Scoville²² in 1919. It is placed in group E and its antigenic formula is III.X.XXVI: e,h : 1,6. Bergey, et al.²³ state that *S. anatum* frequently occurs in association with *S. typhi murium*.

S. typhi murium, a natural pathogen of rodents, was first isolated from a mouse in 1890 by Loeffler and given the name of *B. typhi murium*. In 1898, DeNobele²⁴ recorded a case of gastroenteritis caused by this organism under the name of *S. aertrycke*. The Kauffmann-White schema places this organism in group B with the antigenic structure [I].IV.[V].XII: i: 1,2,3. With the exception of *S. schottmuelleri*, it is the most widely distributed of all the Salmonella.²⁵

Edwards and Bruner²⁶ have demonstrated that each one of the Salmonella found in this outbreak can produce symptoms in most warm-blooded animals.

Clinically, the course of the disease in all cases was of short duration. The symptoms of gastroenteritis disappeared, but the stool cultures were positive for one or more Salmonella at repeated intervals. It is notable that in the 6 cases in which all three species were found none was more acutely ill than any other case. When Salmonella invade the blood stream, however, the symptomatology becomes much more acute. Jager and Lamb²⁷ reported 2 cases of *S. oranienburg* bacteremia. Since the infection involved in this outbreak was apparently confined to the gastrointestinal tract, blood cultures were not taken.

The importance of serologic tests using the patients' homologous antigen proved of great value in our study. The titer for *S. oranienburg* was high and persistent. At no time was there a significant titer for *S. typhi murium* or *S. anatum*. Thus, *S. oranienburg* was the one organism which caused an antibody reaction. Mosher, et al.²⁸ observed no significant antibody titer in patients involved in an outbreak of enteritis due to *S. typhi murium*. Kross and Schiff,²⁹ however, found an agglutination titer of 1:1,600 in a case of *S. typhi murium* enteritis which simulated appendicitis. Other authors have found blood titers for members of the Salmonella group, although it appears that a titer for specific agglutinin is not always produced following an enteric Salmonella infection. Jager and Lamb²⁷ reported a titer for *S. oranienburg*.

The occurrence of Salmonella carriers has been described by Rubenstein, Feemster and Smith.³⁰ Mosher, et al.²⁸ demonstrated *S. typhi murium* in stools of convalescent carriers for eighteen weeks. In our cases *S. typhi murium* was not recovered after the fifth week. This may be due to the influence of sulfa-

guanidine or to the mechanical removal of the organisms in the course of time. *S. anatum* was first identified in the stools during the third week. In all, forty-four patients were found to harbor this organism. Since the patients were isolated and the organism was obtained from patients on different wards, secondary contamination was unlikely. The preponderance of *S. oranienburg* and *S. typhi murium* colonies, however, increased the likelihood of these organisms being fished and studied for identification. The possibility of fewer *S. anatum* organisms originally ingested should not be overlooked. Back* states that the smaller the number of organisms swallowed, the longer the incubation period, and that during the acute symptoms one may obtain practically pure cultures. *S. anatum* persisted for eleven weeks, and *S. oranienburg* for thirteen weeks.

The carrier state is a source of danger to the individual harboring the organism and as the origin of outbreaks. It was with this in mind and in reference to the increased hazard where persons have been congregated that these patients were kept in the hospital until three consecutive negative stools were obtained. The actual hazard has been demonstrated by Hardy and Watt²¹ in the case of fingernail studies in which *Shigella* organisms were recovered. Fingernail studies performed on twelve carriers positive for *S. oranienburg* were negative.

Two sulfonamides were employed in an attempt to eliminate these pathogens. Sulfaguanidine was administered early in the course of the infection and may have been effective in the removal of *S. typhi murium*. There was little or no effect noted with regard to the number of patients whose stools were persistently positive for *S. oranienburg* and *S. anatum*.

When succinyl sulfathiazole was given these patients, the convalescent carrier state was markedly curtailed. Kirby and Rantz²² used this sulfonamide with good results in the treatment of dysentery carriers, but found it to be of no value in the treatment of typhoid carriers. Poth, et al.²³ reported prompt recovery of bacillary dysentery carriers following the administration of sulfasuxidine in small dosages. At the end of the first course of this drug, consisting of 12 Gm. over a period of three days, nearly all of the patients' stools were cleared of *S. anatum*. This small dose was insufficient to produce a marked effect on *S. oranienburg*. Barker²⁴ recommends a dosage of 0.25 Gm. per kilogram for 5 to 7 days to eliminate dysentery bacilli in the stools of carriers. Following the second course of succinyl sulfathiazole, consisting of 75 Gm. over a 5-day period, there was a notable decrease of *S. oranienburg* carriers. The bacteriostatic effect of this sulfonamide was noted culturally and is presented graphically in Fig. 1.

SUMMARY

1. An outbreak is reported involving 115 cases of acute gastroenteritis in military personnel eating at the same mess.

2. The causative organisms identified on stool culture were *S. oranienburg* in 100 (86.9 per cent) cases, *S. typhi murium* in 27 (23.5 per cent) and *S. anatum* in 44 (38.2 per cent).

3. The convalescent carrier nature of the organisms was determined by frequent stool examinations. *S. oranienburg* persisted for 13 weeks, *S. anatum* for 11 weeks and *S. typhi murium* for 5 weeks.

4. Etiological agents were identified culturally, biochemically, and serologically.

5. A high antibody titer for *S. oranienburg* was demonstrated by blood serum agglutinations.

6. Succinyl sulfathiazole in adequate dosage had a bacteriostatic effect on *S. oranienburg* and *S. anatum*, aiding in the termination of the carrier state.

REFERENCES

1. Dunham, G. C.: Military Preventive Medicine, ed. 3, Harrisburg, 1940, Military Publishing Co., pp. 183-187.
2. Simmons, J. S.: Laboratory Methods of the United States Army, ed. 4, Philadelphia, 1935, Lea & Febiger, p. 165.
3. Littman, M. L.: Rapid Identification of Enteric Pathogenic Bacteria, War Medicine 4: 31, 1943.
4. Mayfield, C. R., and Gober, M.: Comparative Efficiency of Plating Media for the Isolation of Shigella Dysenteriae, Am. J. Pub. Health 31: 363, 1941.
5. Baltimore Biological Laboratories, Baltimore, Md.
6. Difco Manual, ed. 7, Detroit, 1943, Difco Co.
7. Hardy, A. V., Watt, J., and DeCapito, T.: Studies of the Acute Diarrheal Diseases. VI. New Procedures in Bacteriological Diagnosis, Pub. Health Rep. 57: 521, 1942.
8. Dack, G. M.: Food Poisoning, Chicago, 1943, University of Chicago Press, pp. 100-132.
9. Leifson, E.: New Selenite Enrichment Media for the Isolation of Typhoid and Paratyphoid (Salmonella) Bacilli, Am. J. Hyg. 24: 423, 1936.
10. Saphra, I., and Silberberg, M.: Taxonomy of Salmonella-like Coliform Organisms, J. Immunol. 44: 129, 1942.
11. Kauffmann, F.: Die Bakteriologie der Salmonella-gruppe, Copenhagen, 1941, Einar Munksgaard.
12. Marshall, E. K., Jr., Bratton, A. C., White, H. J., and Litchfield, J. T., Jr.: Sulfanilyl Guanidine: A Chemotherapeutic Agent for Intestinal Infections, Bull. Johns Hopkins Hosp. 67: 163, 1940.
13. Edwards, P. R., and Bruner, D. W.: The Occurrence of Multiple Types of Paratyphoid Bacilli in Infections of Fowls With Special Reference to Two New Salmonella Species, J. Infect. Dis. 66: 218, 1940.
14. Bornstein, S.: Infections With Organisms of the Salmonella Group, New York State J. Med. 42: 163, 1942.
15. Hormaeche, E., Peluffe, C. A., and Aleppa, L.: Las Salmonelas en Patologia Infantil, Internat. III Congress for Microbiology, 1939, p. 242.
16. Schiff, F., and Saphra, I.: Variety of Types in Human Paratyphoid C. Infections, J. Infect. Dis. 66: 97, 1940.
17. Topley, W. W. C. and Wilson, G. S.: The Principles of Bacteriology and Immunity, ed. 2, Baltimore, 1935, Wm. Wood & Co., p. 1235.
18. Hardy, A. V., and Watt, J.: The Acute Diarrheal Disorders, Am. J. Pub. Health 28: 730, 1938.
19. Kauffmann, F.: Neue serologische Typen der Paratyphusgruppe, Ztschr. f. Hyg. u. Infektionskr. 111: 221, 1930.
20. Edwards, P. R.: The Occurrence of Salmonella Oranienburg Type I in an Infection of Quail, J. Bact. 32: 259, 1936.
21. Schiff, F., and Strauss, L.: Occurrence of Several Unusual Types of Salmonella in Human Infections, J. Infect. Dis. 65: 160, 1939.
22. Rettger, L. F., and Seoville, N. M.: Bacterium Anatum N. S., The Etiological Factor in a Widespread Disease of Young Ducklings Known in Some Places as "Keel," J. Infect. Dis. 26: 217, 1920.
23. Bergey, D. H., et al.: Bergey's Manual of Determinative Bacteriology, ed. 5, Baltimore, 1939, Williams & Wilkins Co., p. 452.
24. DeNobele, J.: Du Serodiagnostic dans les Affections Gastro-Intestinales D'Origine Intestinale, Ann. de la Soc. de Med. 77: 281, 1899.
25. Seligmann, E., Saphra, I., and Wassermann, M.: Salmonella Infections in Man, an Analysis of 1,000 Cases Bacteriologically Identified by the New York Salmonella Center, Am. J. Hyg. 38: 226, 1943.
26. Edwards, P. R., and Bruner, D. W.: The Occurrence and Distribution of Salmonella Types in the United States, J. Infect. Dis. 72: 58, 1943.
27. Jager, B. V., and Lamb, M. E.: Sporadic Infections Caused by Salmonella Suipestifer and Salmonella Oranienburg, New England, J. Med. 228: 299, 1943.
28. Mosher, W. E., Jr., Wheeler, S. M., Chant, H. L., and Hardy, A. V.: An Outbreak Due to Salmonella Typhi murium, Pub. Health Rep. 56: 2415, 1941.

29. Kross, I., and Schiff, F.: Pseudo-surgical Syndromes Produced by Salmonella Organisms, *Am. J. Digest. Dis.* 7: 176, 1940
30. Rubenstein, A. D., Feemster, R. F., and Smith, H. M.: Salmonellosis as a Public Health Problem in War Time, Laboratory Section, American Public Health Association Meeting (Oct. 14), 1943.
31. Hardy, A. V., and Watt, J.: Newer Procedures in Laboratory Diagnosis and Therapy in the Control of Bacillary Dysentery, Laboratory Section, American Public Health Association Meeting (Oct. 14), 1943
32. Kirby, W. M. M., and Rantz, L. A.: Treatment of Typhoid and Dysentery Carriers With Succinyl Sulfathiazole, *J. A. M. A.* 119: 615, 1942
33. Poth, E. J., Chenoweth, B. M., and Knott, F. L.: A Preliminary Report on the Treatment of Bacillary Dysentery With Succinyl Sulfathiazole, *J. Lab. & Clin. Med.* 28: 162, 1942.
34. Barker, P. S.: Treatment of Dysentery Carriers With Succinyl Sulfathiazole: Observation on the Minimal Effective Dose, *Am. J. Digest. Dis.* 10: 443, 1943.

4. Etiological agents were identified culturally, biochemically, and serologically.

5. A high antibody titer for *S. oranienburg* was demonstrated by blood serum agglutinations.

6. Succinyl sulfathiazole in adequate dosage had a bacteriostatic effect on *S. oranienburg* and *S. anatum*, aiding in the termination of the carrier state.

REFERENCES

1. Dunham, G. C.: Military Preventive Medicine, ed. 3, Harrisburg, 1940, Military Publishing Co., pp. 183-187.
2. Simmons, J. S.: Laboratory Methods of the United States Army, ed. 4, Philadelphia, 1935, Lea & Febiger, p. 165.
3. Littman, M. L.: Rapid Identification of Enteric Pathogenic Bacteria, War Medicine 4: 31, 1943.
4. Mayfield, C. R., and Gober, M.: Comparative Efficiency of Plating Media for the Isolation of Shigella Dysenteriae, Am. J. Pub. Health 31: 363, 1941.
5. Baltimore Biological Laboratories, Baltimore, Md.
6. Difco Manual, ed. 7, Detroit, 1943, Difco Co.
7. Hardy, A. V., Watt, J., and DeCapito, T.: Studies of the Acute Diarrheal Diseases. VI. New Procedures in Bacteriological Diagnosis, Pub. Health Rep. 57: 521, 1942.
8. Dack, G. M.: Food Poisoning, Chicago, 1943, University of Chicago Press, pp. 100-132.
9. Leifson, E.: New Selenite Enrichment Media for the Isolation of Typhoid and Paratyphoid (Salmonella) Bacilli, Am. J. Hyg. 24: 423, 1936.
10. Saphra, I., and Silberberg, M.: Taxonomy of Salmonella-like Coliform Organisms, J. Immunol. 44: 129, 1942.
11. Kauffmann, F.: Die Bakteriologie der Salmonella-gruppe, Copenhagen, 1941, Einar Munksgaard.
12. Marshall, E. K., Jr., Bratton, A. C., White, H. J., and Litchfield, J. T., Jr.: Sulfanilyl Guanidine: A Chemotherapeutic Agent for Intestinal Infections, Bull. Johns Hopkins Hosp. 67: 163, 1940.
13. Edwards, P. R., and Bruner, D. W.: The Occurrence of Multiple Types of Paratyphoid Bacilli in Infections of Fowls With Special Reference to Two New Salmonella Species, J. Infect. Dis. 66: 218, 1940.
14. Bornstein, S.: Infections With Organisms of the Salmonella Group, New York State J. Med. 42: 163, 1942.
15. Hormaeche, E., Peluffe, C. A., and Aleppa, L.: Las Salmonelas en Patologia Infantil, Internat. III Congress for Microbiology, 1939, p. 242.
16. Schiff, F., and Saphra, I.: Variety of Types in Human Paratyphoid C. Infections, J. Infect. Dis. 66: 97, 1940.
17. Topley, W. W. C. and Wilson, G. S.: The Principles of Bacteriology and Immunity, ed. 2, Baltimore, 1938, Wm. Wood & Co., p. 1235.
18. Hardy, A. V., and Watt, J.: The Acute Diarrheal Disorders, Am. J. Pub. Health 28: 730, 1938.
19. Kauffmann, F.: Neue serologische Typen der Paratyphusgruppe, Ztschr. f. Hyg. u. Infektionskr. 111: 221, 1930.
20. Edwards, P. R.: The Occurrence of Salmonella Oranienburg Type I in an Infection of Quail, J. Bact. 32: 259, 1936.
21. Schiff, F., and Strauss, L.: Occurrence of Several Unusual Types of Salmonella in Human Infections, J. Infect. Dis. 65: 160, 1939.
22. Rettger, L. F., and Seoville, N. M.: Bacterium Anatum N. S., The Etiological Factor in a Widespread Disease of Young Ducklings Known in Some Places as "Keel," J. Infect. Dis. 26: 217, 1920.
23. Bergey, D. H., et al.: Bergey's Manual of Determinative Bacteriology, ed. 5, Baltimore, 1939, Williams & Wilkins Co., p. 452.
24. DeNobele, J.: Du Serodiagnostic dans les Affections Gastro-Intestinales D'Origine Intestinale, Ann. de la Soc. de Med. 77: 231, 1899.
25. Seligmann, E., Saphra, I., and Wassermann, M.: Salmonella Infections in Man, an Analysis of 1,000 Cases Bacteriologically Identified by the New York Salmonella Center, Am. J. Hyg. 38: 226, 1943.
26. Edwards, P. R., and Bruner, D. W.: The Occurrence and Distribution of Salmonella Types in the United States, J. Infect. Dis. 72: 53, 1943.
27. Jager, B. V., and Lamb, M. E.: Sporadic Infections Caused by Salmonella Suipestifer and Salmonella Oranienburg, New England, J. Med. 228: 299, 1943.
28. Mosher, W. E., Jr., Wheeler, S. M., Chant, H. L., and Hardy, A. V.: An Outbreak Due to Salmonella Typhi murium, Pub. Health Rep. 56: 2415, 1941.

slow and several years were required to accumulate enough dried organisms to proceed with chemical extraction and analysis. For information on the nitrogenous constituents of the organism, the nitrogen distribution in the dried bacteria was determined by the Cavett⁸ modification of the Van Slyke⁹ procedure. The ash content was determined by heating to redness in a muffle furnace for six hours. Phosphates were estimated by the Fiske and Subbarow¹⁰ method on acid solutions of the ash. The colorimetric method of Shinohara¹¹ was used to determine cystine, and the Benedict-Denis method was used for total sulfur. The average values obtained are shown in Table I.

TABLE I
BACTERIAL ANALYSIS.

	IC STRAIN* PER CENT OF TOTAL N	CS STRAIN† PER CENT OF TOTAL N	IC STRAIN* PER CENT	CS STRAIN† PER CENT
Amide N	13.16	11.08		
Humin N	4.17	4.09		
Dibasic N	29.52	29.97		
Arginine	8.55	8.20		
Histidine	9.94	1.20		
Lysine	10.45	1.58		
Cystine	0.58	0.7		
Mono- and non-amino N	53.61	51.7		
Amino N	48.10	45.16		
Total N accounted for	100.46	100.6		
Ash			13.91	13.00
Phosphorus			1.95	1.80
Sulfur			0.45	0.47
Nitrogen			14.18	12.79

*Iowa City strain.

†Council Bluffs strain.

EXTRACTION PROCESS

In preliminary experiments phenol and trichloroacetic acid were found unsatisfactory as solvents for isolating the carbohydrate from the dried ground organisms. Diethylene glycol, which had been tried by Morgan,¹² gave good results until dialysis with cellophane was attempted; the carbohydrate passed through the cellophane casing and the yield was lost in the dialysate. Fuller's method¹³ for obtaining polysaccharides from streptococci, employing formamide as the solvent, was found satisfactory.

The dried ground bacteria, usually in 5 or 10 Gm. portions, were heated with 15 volumes of formamide at 150° C. for 20 minutes. After cooling, 2½ volumes of acidified alcohol were added, the precipitate was filtered off and re-extracted twice with 70 per cent alcohol. The combined alcoholic extracts were mixed with an equal volume of acetone and the precipitate which contained the extracted carbohydrate was separated by centrifugation and dissolved in a small amount of water. A portion remained undissolved even after small amounts of dilute acid were added. This insoluble fraction was largely nucleoprotein.

Further separation of the water-soluble material was effected by fractional precipitation with acidified acetone. Two to 5 volumes of acetone precipitated most of the carbohydrate. Addition of 15 volumes of acetone to the supernatant liquid produced a surprisingly large precipitate. Thus three fractions were obtained: nucleoprotein, carbohydrate, and the 15-volume acetone precipitate.

CHEMICAL STUDIES

Chemical studies on the above fractions were confined to the carbohydrate and the 15-volume acetone precipitate, since the amount of nucleoprotein was insufficient for analysis.

Aqueous solutions of the carbohydrate did not reduce Benedict's solution until after hydrolysis. The biuret test was weakly positive. The iodine test for glycogen was negative; the Molisch test was positive in every case. Positive tests for pentose were given by Bial's and the naphthoresoreinol test. According to Tollens' naphthoresoreinol test glycuronic acid was not present. Methyl pentoses were not present as shown by a negative response to the ammonium molybdate test, the Tollens and Rorive modification of the naphthoresoreinol test and spectral reactions. Characteristic osazone crystals were formed as irregular rosettes with smooth broad petals; they were soluble in hot water. These osazones could not be identified with those prepared from pure specimens of several pentoses and hexoses.

Sugar analyses were made by the Somogyi method¹⁴ with the microsugar reagent. Hydrolysis of 5 to 10 mg. samples was carried out by gentle boiling of the mixture on a sand bath under a reflux with 5 c.c. of 0.5N HCl for five to eight hours. The method of Somogyi¹⁵ was used for the determination of fermentable and nonfermentable sugar. Larger samples of the polysaccharide (15 to 30 mg.) were hydrolyzed for this procedure.

Nitrogen was estimated by a semimicro-Kjeldahl method on 5 to 10 mg. of material. Amino nitrogen was determined by the Kendrick and Hanke modifications^{16, 17} of the Van Slyke method on aliquots of acid hydrolysates (0.5N HCl) containing 5 mg. of the polysaccharides.

Ash was determined by igniting 20 mg. samples at bright red heat for six hours in a muffle furnace. Phosphates were estimated by the Fiske and Subbarow¹⁸ method on acid solutions of the ash.

The specific rotation of the preparations was measured on aqueous solutions which were then made 0.5N by the addition of hydrochloric acid and hydrolyzed for six hours, as described previously.

Equivalent weight was obtained by titration of 20 mg. samples with 0.01N NaOH and phenolphthalein as the indicator. The samples were dissolved in approximately 1 c.c. of water.

Since the qualitative tests for pentose and uronic acid are so closely related, a modification of the method of Bowman and McKinnis¹⁹ for the simultaneous determination of pentose and uronic acid was used in the quantitative estimation. The furfural that was formed was measured by Heidelberg's modification²⁰ of the Pervier and Gortner method.²⁰ Uronic acid was not present in the polysaccharides since the formation of barium carbonate never exceeded that of a blank determination. Samples of arabinose (10 mg.) were analyzed for their pentose content by this method. Values of from 9.77 to 10.26 mg. were obtained in six determinations.

The glucosamine content of the polysaccharides was estimated by a combination of the methods of Palmer, Smyth, and Meyer,²¹ and Elson and Morgan.²²

The average values for some of the preparations are shown in Table II.

TABLE II
POLYSACCHARIDE ANALYSIS

	IC STRAIN		CB STRAIN		15-VOLUME ACETONE PRECIPITATE
	FROM 10 GM.	FROM 5 GM.	FROM 10 GM.	FROM 5 GM.	
Reducing sugar after hydrolysis	18.27	21.50	19.20	19.84	1.50*
Nonfermentable sugar	15.82	18.60	17.48	17.60	1.15
Fermentable sugar	2.45	2.90	1.72	2.24	0.35
Pentose	17.13	18.97	19.61	18.68	6.16
Total nitrogen	8.18	7.08	6.50	6.92	21.40
Amino nitrogen	3.07	2.41	2.99	2.47	9.55
Per cent of total nitrogen	37.50	34.10	46.00	35.70	44.60
Glucosamine	8.97	7.20	6.70	9.57	4.63
Ash	2.44	2.84	4.64	4.17	3.05
Phosphorus	1.06	1.22	1.09	1.00	0.83
Acid equivalent N/100 NaOH	463.00	370.00	407.00	370.00	637.00
[α] _D 27 before hydrolysis	+8.02	-8.60	2.81	+7.63	-29.30†
[α] _D after hydrolysis	+15.27	+12.93	+13.50	+14.11	+33.70†

*Hydrolyzed with 4N HCl.

†[α] _D 35TABLE III
FLOCCULATION TESTS OF CARBOHYDRATE FRACTIONS WITH THEIR ANTISERA

CARBOHYDRATE ANTISERA	DILUTION OF ANTIGEN				
	10-1	10-2	10-3	10-4	10-5
IC antigen					
IC-1	+	+	+	-	-
IC-2	+	+	-	-	-
Normal serum	-	-	-	-	-
CB antigen					
CB-1	+	+	+	-	-
CB-2	+	+	+	-	-
Normal serum	-	-	-	-	-

ANTIGENIC REACTIONS

So little of the carbohydrate was left after the chemical investigation was finished that amounts used and numbers of animals employed were limited. Two rabbits were inoculated with each of the polysaccharides once a week. The initial dose was 1 mg.; in each of the next four weeks they received 2 mg., and after a rest of one week each received 1 mg. This made 10 mg. of carbohydrate that each animal received. The rabbits were bled the seventh week.

Flocculation tests were again used to determine the results of the immunization. Tenfold dilutions of each of the polysaccharide preparations were made and the antiserum held constant at a 1:5 dilution. The two components were used in 0.5 c.c. amounts. The tests were incubated in the cold room overnight and at room temperature for a few hours the next day. The results appear in Table III.

One month after immunization the animals were bled and tested again. These results were more promising and appear in Table IV.

There were available some antisera prepared by injection of 3-hour cultures of whole organisms of other strains of pathogenic staphylococci including the two strains investigated here. These strains were designated IC, CB, X, and Y. Flocculation tests were set up with the diluted polysaccharide antigens from IC and CB strains and antiserum to all four strains of whole organism.

TABLE IV

FLOCCULATION TESTS OF CARBOHYDRATE FRACTIONS WITH THEIR ANTISERA*

CARBOHYDRATE ANTISERA	DILUTION OF ANTIGEN				
	10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵
	IC antigen				
IC-1	+	+	+	+	-
IC-2	+	+	+	+	-
Normal serum	-	-	-	-	-
	CB antigen				
CB-1	+	+	+	+	-
CB-2	+	+	+	-	-
Normal serum	-	-	-	-	-

*These antisera were taken 30 days after last immunization of animals.

TABLE V

FLOCCULATION TESTS OF CARBOHYDRATE FRACTIONS WITH VARIOUS ANTISERA PREPARED BY USE OF WHOLE ORGANISMS AS ANTIGEN

CARBOHYDRATE ANTISERA	DILUTION OF ANTIGEN					
	10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶
	IC antigen					
IC	+	+	+	+	+	+
CB	+	+	+	+	+	+
X	+	+	+	+	+	+
Y	+	+	+	+	+	+
Normal serum	-	-	-	-	-	-
	CB antigen					
IC	+	+	+	+	+	+
CB	+	+	+	+	+	+
X	+	+	+	+	+	+
Y	+	+	+	+	+	+
Normal serum	-	-	-	-	-	-

TABLE VI

FLOCCULATION TESTS OF SUPERNATANT OF CULTURES WITH ANTISERUM TO CARBOHYDRATE FRACTIONS

CARBOHYDRATE ANTISERA	DILUTION OF SUPERNATANT		
	10 ⁻¹	10 ⁻²	10 ⁻³
	IC supernatant		
IC-1	+	+	+
IC-2	+	+	+
Normal serum	-	-	-
	CB supernatant		
CB-1	+	+	+
CB-2	+	+	+
Normal serum	-	-	-

These antisera had been preserved in sealed ampoules for more than six months at time of use. The outcome of these tests is found in Table V.

Next the supernatant of the cultures used for producing the polysaccharide was tested against the antiserum to the polysaccharide. Dilutions of the supernatant of 24-hour cultures of IC and CB in dextrose meat infusion broth were diluted from 10⁻¹ to 10⁻⁵ and the antisera were used without dilution. Equal volumes of the components were employed and the same incubation procedures utilized previously were followed. These findings are recorded in Table VI.

DISCUSSION

Based on the analysis of whole bacteria, there appeared to be no demonstrable difference between the two strains of staphylococci.

The method of preparation of the polysaccharide does not appear to produce a protein-free product. A slight biuret test, however, does not indicate a large amount of protein. The rather high nitrogen content may indicate contamination of the product by protein. On the other hand, the amino nitrogen accounts for approximately 40 per cent of the total nitrogen which would suggest nitrogen-containing material other than protein. The amino sugar present could account for only a small proportion of the total amino nitrogen unless it was altered in the process of preparation or hydrolysis.

The values obtained for reducing sugar after hydrolysis and for fermentable and nonfermentable sugars are in close agreement with those reported by Julianelle and Wieghard³ for a pathogenic strain of staphylococcus. The presence of a large proportion of nonfermentable reducing sugar suggested the possibility of a pentose, but the above authors reported a negative qualitative test for this substance. Every preparation of the carbohydrate in this investigation gave strongly positive pentose tests. As little as 0.03 mg. gave a positive Bial's test. The values obtained in the quantitative estimation of pentoses accounted for slightly more than the nonfermentable sugar of the polysaccharide. It is not improbable that the amino sugar may be present as an amino pentose.

The polysaccharides were characterized by a low dextrorotation after hydrolysis. The material that was precipitated by 15 volumes of acetone had a higher rotation with little change on hydrolysis. This material gave abnormally high total and amino nitrogen values and in every case gave a value for pentose although it exhibited no reducing sugar after hydrolysis with 0.5N HCl. When hydrolyzed with 4N HCl this preparation yielded a small quantity of reducing sugar which was mainly nonfermentable.

Experiments on the antigenic capacity of the carbohydrate preparations were of necessity limited because of the small amount of the polysaccharide available. It was possible to show that a relationship existed between the polysaccharide antigen and antisera developed against the whole organism. Four different antisera to the whole organism of the pathogenic group reacted with each of the carbohydrate preparations to at least a dilution of 1:1,000,000 of the antigen. The reaction might have been even more delicate if the antisera had been fresh.

The reaction between the polysaccharide antigen and its antiserum produced in rabbits indicated that the carbohydrate was antigenic to some degree. A flocculation test set up immediately after the completion of immunization showed that the antigen could be diluted at least to 1:1,000 and give a visible reaction with the antiserum. The antigen has nitrogen in its complex but, as discussed previously, it is not determined in what form it occurs, whether as a contaminating protein or as amino groups in the sugar molecule. When the sera from the immunized animals were again tested after thirty days, they demonstrated an increased antibody content; dilutions of 1:10,000 gave flocculation.

It was interesting to find that cultures of these pathogenic staphylococci gave off to the medium sufficient of the antigenic substance to react with the carbohydrate antiserum. This reaction could possibly be used in diagnostic

procedures to distinguish between pathogenic and nonpathogenic strains. Julianelle and Wieghard³ have already shown that there is an antigenic difference between the two divisions.

SUMMARY

1. A polysaccharide, containing some nitrogen, has been obtained from pathogenic strains of staphylococci by use of formamide as an extractive.

2. This polysaccharide yields a pentose on hydrolysis. The osazone crystals of this pentose have not been identified.

3. The polysaccharide is antigenic and reacts in flocculation tests with its own antiserum, and with antisera against whole organisms.

4. The culture medium in which these strains of pathogenic staphylococci were grown contained enough polysaccharide to give a flocculation test with the antisera produced with the pure polysaccharide.

5. There were no demonstrable differences between the two strains of staphylococci used.

The authors wish to thank Dr. W. M. Hale, chairman of the Department of Bacteriology, University of Iowa, for facilities, media, and counsel in the accomplishment of a part of this study in that department.

REFERENCES

1. Goadby, K. W.: Bacterial Proteins: The Presence of Alcohol-Soluble Proteins in Bacteria, *Proc. Roy. Soc. London* s. B. 102: 137, 1927.
2. Goadby, K. W.: The Antigenic Properties of Different Fractions of *Staphylococcus*, *J. Path. & Bact.* 35: 657, 1932.
3. Julianelle, L. A., and Wieghard, C. W.: The Immunological Specificity of Staphylococci. I. The Occurrence of Serological Types. II. The Chemical Nature of the Soluble Specific Substances. III. Interrelationships of Cell Constituents, *J. Exper. Med.* 62: 11, 23, 31, 1935.
4. Hoffstadt, R. E., and Clark, W. M.: The Chemical Composition and Antigenic Properties of Fractions of the Smooth and Rough Strains of *Staphylococcus aureus*, *J. Infect. Dis.* 62: 70, 1938.
5. Verwey, W. F.: A Type-Specific Antigenic Protein Derived From the *Staphylococcus*, *J. Exper. Med.* 71: 635, 1940.
6. Lyons, C.: Antibacterial Immunity to *Staphylococcus pyogenes*, *Brit. J. Exper. Path.* 18: 411, 1937.
7. Fellowes, O. N.: Unpublished data.
8. Cavett, W. J.: A Modification of the Van Slyke Nitrogen Distribution Method, *J. Biol. Chem.* 95: 335, 1932.
9. Van Slyke, D. D.: A Method for Quantitative Determination of Aliphatic Amino Groups. Applications to the Study of Proteolysis and Proteolytic Products, *J. Biol. Chem.* 9: 185, 1911.
10. Fiske, C. H., and Subbarow, Y.: The Colorimetric Determination of Phosphorus, *J. Biol. Chem.* 66: 375, 1925.
11. Shinohara, K.: The Determination of Thiol and Disulfide Compounds, With Special Reference to Cysteine and Cystine. V. A Critical Study of Cystine Determination by Sulfite and Phospho-18-Tungstic Acid Reagent, *J. Biol. Chem.* 112: 683, 1935-36.
12. Morgan, W. T. J.: Studies in Immuno-Chemistry. II. The Isolation and Properties of a Specific Antigenic Substance From *B. dysenteriae* (Shiga), *Biochem. J.* 31: 2003, 1937.
13. Fuller, A. T.: The Formamide Method for the Extraction of Polysaccharides From Hemolytic Streptococci, *Brit. J. Exper. Path.* 19: 130, 1938.
14. Somogyi, M.: A Reagent for the Copper-Iodometric Determination of Very Small Amounts of Sugar, *J. Biol. Chem.* 117: 771, 1937.
15. Somogyi, M.: The Distribution of Sugar in Normal Human Blood, *J. Biol. Chem.* 78: 117, 1928.
16. Kendrick, A. B., and Hanke, M. E.: The Use of Iodine and Other Modifications in the Van Slyke Manometric Amino Nitrogen Method, *J. Biol. Chem.* 117: 161, 1937.
17. Kendrick, A. B., and Hanke, M. E.: The Effect of Iodine and Mercury on Amino Nitrogen Values With Nitrous Acid, *J. Biol. Chem.* 132: 739, 1940.

18. Bowman, J. R., and McKinnis, R. B.: A Study of the Pentose and Uronic Acid Content of Orange Albedo, and Arabino-Galacturonic Acid Derived From Orange Pectin, *J. Am. Chem. Soc.* 52: 1209, 1930.
19. Heidelberger, M., and Kendall, F. E.: Specific and Nonspecific Polysaccharides of Type IV Pneumococcus, *J. Exper. Med.* 53: 625, 1931.
20. Pervier, N. C., and Gortner, R. A.: The Estimation of Pentoses and Pentosans. I. The Formation and Distillation of Furfural. II. The Determination of Furfural, *J. Ind. Eng. Chem.* 15: 1167, 1255, 1923.
21. Palmer, J. W., Smyth, E. M., and Meyer, K.: On Glycoproteins. IV. The Estimation of Hexosamine, *J. Biol. Chem.* 119: 491, 1937.
22. Elson, L. A., and Morgan, W. T. J.: A Colorimetric Method for the Determination of Glucosamine and Chondrosamine, *Biochem. J.* 27: 1824, 1933.

SERIAL BIOPSY STUDIES OF THE EFFECTS OF ESTROGENS ON THE LIVER

CHEMICAL AND MORPHOLOGIC RESPONSES TO DIETHYLSTILBESTROL AND ESTRADIOL

HAROLD K. ROBERTS, M.D., ELSON B. HELWIG, M.D., ROBERT ELMAN, M.D.,
AND CYRIL M. MACBRYDE, M.D., ST. LOUIS, MO.

SINCE the introduction of the synthetic estrogen, diethylstilbestrol, a number of reports on the estrogenic potency and the toxicology of this substance have appeared. In some of the investigations the properties of the synthetic estrogen were compared with the properties of the estrogens derived from natural sources. It is apparent from these studies that the quality of estrogenic activity of diethylstilbestrol is comparable to that of the natural estrogens. Furthermore, it has been shown in animals that these two different types of estrogenic substances are capable of producing similar possibly toxic changes in various organs and tissues.¹ Because an appreciable number of women receiving the synthetic estrogen diethylstilbestrol have experienced nausea and vomiting, and since in a few cases it has been suspected of causing jaundice, attention has been focused on the liver. No definite evidence has been produced by studies of hepatic function in man to show that diethylstilbestrol damages the liver. The results obtained following the administration of diethylstilbestrol to animals permit divergent conclusions in regard to the effect on the liver.²⁻⁶ The hepatic changes have been variously interpreted as fatty degeneration and hydropic degeneration, fatty degeneration and toxic necrosis, hepatitis, and glycogen deposition. Some of the papers include reports of parallel studies with estrogens derived from natural sources, with similar results.

In considering the toxicity both of diethylstilbestrol and of the natural estrogens, it must be recognized that the dosage per unit of body weight employed in many of the experiments on animals exceeded the therapeutic range used in man. Furthermore, there are apparent differences in species susceptibility to these substances. An alteration of the bone marrow and the formed elements of the peripheral blood is easily produced by the administration to dogs of large doses either of diethylstilbestrol or of the natural estrogens.^{1, 7} Similar changes are much more difficult to produce in the rat⁸ and have not been observed in the monkey.^{9, 10} Therefore it seems possible that estrogenic substances might influence the histologic picture and the metabolism of the liver of different species of animals in different manners. In a previous study⁵ two of us gave large doses of natural and synthetic estrogens to dogs. Hepatic changes were caused by both estrogens, consisting of mild fatty degeneration and hydropic degeneration. There was considerable variation in the amount of glycogen

From the Departments of Medicine, Pathology and Surgery, Washington University School of Medicine, St. Louis, Missouri.

Received for publication July 29, 1944.

present in different livers as determined by Best's carmine stain. No correlation could be established between the degree of fatty degeneration and hydropic degeneration and the amount of glycogen present. Tengne,⁶ however, has shown that the amount of glycogen in the livers of rats increases following the administration of estrogenic substances. To determine more accurately the nature of the changes observed in the livers of dogs following the administration of estrogens, we have done chemical and histological studies on samples of liver obtained both before and after the administration of diethylstilbestrol and of estradiol.*

MATERIAL AND METHODS

The studies reported were performed on eight healthy, mature dogs, 5 females and 3 males, weighing from 6 to 19 kilograms. Four of the animals, 3 females and 1 male, were used as controls. The dogs were given a diet of Purina Dog Chow. Complete counts of the formed elements of the peripheral blood were made two or three times a week.

At the termination of the control period each animal was weighed (fasting), and blood was drawn from the external jugular vein for determinations of the hematocrit, total protein, albumin, and globulin. Each animal was then anesthetized by intravenous sodium pentobarbital (0.5 mg. per kilogram of body weight). After ligation of the end of an accessible lobe of the liver a small specimen was quickly excised. One part of the specimen was used for quantitative determinations of glycogen, fat and total nitrogen and another part was used for histologic studies. Small samples of tissue were fixed in absolute alcohol and examined for glycogen by Best's carmine stain, and in solution of formaldehyde U.S.P. diluted 1:10 and examined for fat by the scarlet red stain. In addition comparable samples of tissue were fixed in a solution of Zenker's acetic acid and stained with eosin and methylene blue (methylthionine chloride U.S.P.).

The animals were divided into four groups of two each according to the type of intramuscular injections given. A dose of 1.66 mg. of estradiol dipropionate was considered to be approximately equivalent estrogenically to 5 milligrams of diethylstilbestrol dipropionate.

Group I—One male and one female dog, numbers 1 and 2, were given daily injections of 5 mg. of diethylstilbestrol dipropionate in 1 c.c. of olive oil. On the seventeenth day of the injections, a count of the blood disclosed a moderate thrombocytopenia. On the following morning biopsy specimens of the liver were obtained, and immediately thereafter complete autopsies were performed.

Group II—Dogs 3 and 4, male and female respectively, served as the control animals for the Group I dogs. Each dog received 1 c.c. of sterile olive oil for 17 days, then biopsy specimens of the liver were obtained, followed by complete autopsies.

Group III—One male and one female dog, numbers 5 and 6, were given daily injections of 1.66 mg. of alpha estradiol dipropionate in 1 c.c. of sesame

*The diethylstilbestrol dipropionate was supplied by the Department of Medical Research, Winthrop Chemical Company, New York. Estradiol was furnished by the Medical Research Division of the Schering Corporation, Bloomfield, New Jersey, and by Roche-Santon, Inc., Nutley, New Jersey.

oil. By the sixteenth day a marked thrombocytopenia had occurred. On the following morning the second biopsy of each liver was performed. After the biopsy an autopsy was done.

Group IV—Dogs 7 and 8, both females, were injected daily with 1 c.c. of sterile sesame oil for 16 days, and served as control animals for Group III.

Determinations of the percentage of glycogen in the liver were done by the method of Good, Kramer, and Somogyi.¹¹ A modification of Bloor's method described by Elman and Heifetz¹² was used for the determinations of fat. The wet liver sample was weighed, and the total nitrogen was measured by the method of Sobel, Yuska and Cohen.¹³ Hematocrit determinations and measurement of serum albumin and globulin were obtained before and after each period of medication.

RESULTS

1. Chemical Changes.—In the table the results of the chemical analyses of the specimens of liver removed from the eight dogs prior to and after the administration either of the estrogenic substances or of the oily vehicles are presented.

2. Hematocrits and Blood Proteins.—The hematocrit and blood protein values obtained before and after the administration of the estrogens revealed no significant alterations.

3. Anatomical Changes.—The alterations in the gross appearance of the livers of the animals receiving the estrogenic substances were not striking. No distinction in the gross appearance of the livers could be made on the basis of the type of estrogenic substance administered. The site of the previous operation in each liver did not appear unusual.

For the microscopic examination each specimen of liver was divided into three contiguous samples. One was stained with eosin and methylene blue, another with Best's carmine and the third with scarlet red. By this method the amount and distribution of histologically demonstrable fat and glycogen were compared with the histologic picture in each specimen. In addition, comparisons of the histologic content and distribution of fat and glycogen and the histologic appearance were made among the different specimens.

LIVER BIOPSY SPECIMENS DURING CONTROL PERIOD

The microscopic appearance of the control specimens of liver removed surgically in the premedication periods was essentially normal. The eosin and methylene blue stain revealed a definite vesicular appearance in some of the hepatic cells. In most instances the hepatic cells of the central zone were less vesicular than those of the middle and in particular those of the peripheral zone. A comparison of the sections stained with eosin and methylene blue with sections from the same specimen stained with Best's carmine showed deposits of glycogen in the cells with a vesicular cytoplasm. Of the control specimens, the liver of Dog 1 appeared to contain the most and the liver of Dog 3 the least amount of glycogen. No sharp distinction could be made concerning the relative amount of glycogen present in the other six normal specimens of liver. The vesicular character of the cytoplasm of the hepatic cells as noted in the sections

CHEMICAL ANALYSES OF LIVER BIOPSY SPECIMENS FROM DOGS RECEIVING DIETHYLSTILBESTROL OR ESTRADIOL

DOG NO.	WT. KG.	MEDICATION	DAILY DOSE	NO. DAYS INJECTED	INTERVAL BETWEEN BIOPSIES—DAYS	LIVER ANALYSIS BEFORE (A) AND AFTER (P) INJECTIONS				
						GLYCOGEN %	DIFFER- ENCE %	FAT %	DIFFER- ENCE %	TOTAL NITROGEN %
Group I 1 2	6.2	Diethyl stilbestrol dipropionate	5 mg.	17	21	(a) 6.28 (p) 4.18	-2.10	7.8	-4.2	2.50
	7.4	Diethyl stilbestrol dipropionate	5 mg.	17	21	(a) 5.34 (p) 9.19	+3.85	2.4 2.5	+0.1	2.68 2.87 2.51
Group II 3 4	5.6	Olive oil	1 c.c.	17	21	(a) 0.58 (p) 3.77	+3.19	3.2	-1.6	3.09
	6.6	Olive oil	1 c.c.	17	21	(a) 3.85 (p) 2.78	-1.07	3.6 2.1	-0.8	2.90 3.03 3.12
Group III 5 6	19.5	Alpha estradiol dipropionate	1.66 mg.	16	26	(a) 12.32 (p) 2.42	-10.9	1.2	+1.2	2.07
	15	Alpha estradiol dipropionate	1.66 mg.	16	26	(a) 4.85 (p) 3.06	-1.79	2.32 2.5	0.02	2.85 2.83 2.85
Group IV 7 8	15	Sesame oil	1 c.c.	16	26	(a) 0.98 (p) 4.7	+3.32	3.9	-1.8	3.31
	17.5	Sesame oil	1 c.c.	16	26	(a) 1.1 (p) 10.7	+9.6	2.1 6.1 1.4	-1.7	2.91 3.23 2.33

stained with eosin and methylene blue was not entirely due to the deposition of glycogen, since the tissue stained with scarlet red showed a minimal number of fine fat droplets in the cytoplasm of the hepatic cells. The droplets of fat tended to be larger and more numerous in the regions of the portal canals and the central veins. The control specimen of liver of Dog 1 and of Dog 3 appeared to contain the most fat. The other six control specimens contained a minimal amount of histologically demonstrable fat.

The microscopic appearance of the control specimen of liver removed surgically in the premedication period of each dog was compared with the microscopic appearance of the liver following the period of medication.

LIVER BIOPSY SPECIMENS AFTER DIETHYLSTILBESTROL

The two dogs in Group I received 5 mg. of diethylstilbestrol dipropionate daily for seventeen days. After the injections the liver of Dog 1 showed a slight increase in stainable glycogen and a moderate decrease in stainable fat. The decrease in fat was chiefly due to a decrease in the number of the larger fat droplets. There were rare vacuoles in the hepatic cells which failed to stain either as fat or glycogen. In the periportal spaces there was a slight increase in the number of mononuclear cells. After the diethylstilbestrol injections the liver of Dog 2 exhibited an appreciable increase in the content of stainable glycogen in practically all of the hepatic cells. There appeared to be a minimal increase in the amount of stainable fat in most of the parenchymal cells.

The two dogs in the control Group II received 1 c.c. of olive oil daily for seventeen days. After the administration of olive oil to Dog 3 the liver showed an appreciable increase in the amount of stainable glycogen and fat. In neither instance was the liver otherwise unusual.

LIVER BIOPSY SPECIMENS AFTER ESTRADIOL

The two dogs in Group III received 1.66 mg. of alpha estradiol dipropionate daily for sixteen days. The liver of Dog 5 exhibited a slight decrease in the stainable glycogen content after the estrogen injections. This decrease occurred in the middle and peripheral zones where glycogen was noted in the normal control specimen. The central zones were not appreciably involved either before or after the medication. The amount of stainable fat was scanty and no change could be discerned. Rarely vacuoles were noted which failed to stain either as glycogen or fat. After the administration of the estrogenic substance to Dog 6 the liver showed a minimal increase in the amount of stainable glycogen; however, the glycogen was confined chiefly to the cells of the peripheral and central zones. There was no alteration in the content of stainable fat.

The two dogs in control Group IV received injections of 1 c.c. of sesame oil daily for sixteen days. After the administration of sesame oil to Dog 7 the liver showed a moderate increase in the amount of stainable glycogen distributed rather uniformly throughout all zones of the hepatic lobules. No change was observed in the amount of stainable fat. After the sesame oil injections the liver of Dog 8 showed a definite increase in stainable glycogen. The glycogen was equally distributed throughout each lobule except for a somewhat smaller

amount immediately adjacent to the central veins. No change was noted in the content of stainable fat.

DISCUSSION

These observations reveal no consistent effect of estradiol or of diethylstilbestrol upon the livers of dogs. No correlation was established between administration of the estrogenic substances and chemical and histochemical changes in the liver.

A comparison of the values of hepatic glycogen obtained by chemical analysis before and after the administration of diethylstilbestrol dipropionate revealed a variable result. Following the injections there was a rise in the hepatic glycogen in one instance and a drop in another. Following the administration of alpha estradiol dipropionate there was a drop in the amount of hepatic glycogen in both examples; however, in one the drop was not appreciable. The hepatic glycogen in the control dogs before and after the administration either of sesame oil or of olive oil likewise showed a variation. It appears that the amount of glycogen in the liver may either increase or decrease following the administration of the estrogenic substances or of the oils alone.

A comparison of the values of the hepatic fat before and after the administration of the estrogenic substances showed inconsistent results. One of the two dogs receiving diethylstilbestrol showed a decrease in the amount of hepatic fat. The amount of fat in the liver of the other dog was unaltered. The two dogs injected with alpha estradiol dipropionate exhibited a rise in the hepatic fat in one instance and no change in the other. The four control dogs receiving either olive oil or sesame oil showed a variable but usually slight drop in the amount of hepatic fat. Thus, the administration of the estrogenic substances did not produce consistent changes in the amount of hepatic fat.

The total nitrogen content of the livers before and after the administration of either of the estrogenic substances or of the oils showed no appreciable change.

Investigators have reported evidence of hepatic damage in animals receiving large doses of estrogenic substances. In a previous paper two of us² described the occurrence of mild fatty degeneration and hydropic degeneration in the livers of dogs given equivalent doses of "natural" and synthetic estrogens. Furthermore, these livers contained a variable but usually scanty amount of glycogen as determined by Best's carmine stain. These observations were in disagreement with those of Teague,⁶ who studied the effect of diethylstilbestrol and of estradiol on the livers of rats. He concluded that the vacuolization of the hepatic cells was due to an accumulation of glycogen and not to fatty degeneration. Recently Elman and Heifetz¹² demonstrated extensive vacuolization of the parenchymal cells of the liver in dogs maintained upon a protein deficient diet. Chemical analyses and stained sections of the livers showed the vacuolization not to be due to the accumulation of fat or of glycogen. In the present study, in order to determine the nature of the microscopic changes in the liver, concomitant chemical and histochemical analyses were done. The results of the chemical analyses showed little change in the value of the total hepatic nitrogen, after the administration of the estrogenic substances. A histologic comparison rarely showed a vacuole in the hepatic cells which failed to

stain either for fat or for glycogen. On the basis of the work reported by Elman and Heifetz it does not seem probable that the hepatic vacuoles in the present study were due to a deficiency of protein. In the previous study⁶ of the effect of estrogens upon the liver, vacuoles were encountered which did not stain either with scarlet red or with Best's earmine. However, the dogs received larger amounts of estrogenic substance over a longer period of time than did the dogs in the present investigation, and death was often preceded by a period of cachexia.

In the present study there was no exact correlation between the quantitative chemical content of glycogen in the liver and the amount as determined by histochemical estimation. The lowest value of hepatic glycogen determined by chemical analysis occurred in a liver which also appeared to have the least glycogen by histochemical estimation. It was rarely possible to estimate correctly the amount of glycogen in the liver by histochemical means in that group of livers which contained the most glycogen. This conclusion is essentially in agreement with the observations of Grafflin, Marble, and Smith.¹⁴ These authors made histologic estimations of the content of glycogen in the livers of guinea pigs and compared the result with the chemically determined glycogen content of each liver. They concluded that histologic estimation of the hepatic glycogen was at best only approximate. In the present study there was some variation in the occurrence of the glycogen in different areas of the same sample of tissue. This fact may in part explain the unreliability of the histologic estimations of hepatic glycogen.

A comparison of the histologic estimation of the hepatic fat with the amount present as determined by quantitative analysis shows only approximate correlation. Those specimens of liver in which fat appeared more abundant by histologic estimation generally showed larger amounts by quantitative analysis. When the amount of fat was low, as determined chemically, the histologic estimation frequently showed little correlation with the results of the chemical analysis.

Janes and Nelson¹⁵ and Griffiths, Marks and Young¹⁶ have demonstrated by chemical analysis an increase of the hepatic glycogen following the administration of estrogenic substances to rats. This result is in accord with Teague's observation of an histologic increase of hepatic glycogen in the rat following estrogenic medication. The results of these workers are at variance with observations in the present study on dogs. It is known that estrogens may produce certain blood changes in one species of animal, but little or no corresponding effects in other species. It is possible that the metabolism of the liver is also affected differently in various species of animals following the administration of estrogenic substances. The results of this study indicate that in dogs neither hepatic glycogen nor hepatic fat are consistently altered by the amounts of estrogens and the durations of administration used in these experiments.

SUMMARY AND CONCLUSIONS

1. Hepatic glycogen, fat and nitrogen as determined by chemical analysis of biopsy specimens showed no significant alteration following the administration to dogs of diethylstilbestrol dipropionate or of alpha estradiol dipropionate.

2. The histologic estimation of the amount of hepatic glycogen and hepatic fat could be only approximately correlated with the chemical analyses.

3. Morphologic changes such as hydropic or fatty degeneration of the liver, which we have previously found in dogs receiving large doses of synthetic or natural estrogens, were not observed in this study. This may be explained by the absence of the profound blood changes previously induced.

The authors express appreciation to Mrs. Harriet Wolf Daves and to Miss Elizabeth Houghton for valuable technical assistance.

REFERENCES

1. Castrodale, D., Bierbaum, Olga, Helwig, E. B., and MacBryde, C. M.: Comparative Studies of the Effects of Estradiol and Stilbestrol on the Blood, Liver and Bone Marrow, *Endocrinology* 29: 363, 1941.
2. Loeser, A.: Untersuchungen über die Pharmakologie und Toxikologie synthetischer Brunststoffe (4-4' Dioxy-alpha beta-Diäthylstilben), *Ztschr. f. d. ges. exper. Med.* 105: 430, 1939.
3. Arnold, O., and Hamperl, H.: Untersuchungen zur Toxikologie des 4,4'-Dioxy- α , β -Diäthylstilbens, *Arch. f. exper. Path. u. Pharmacol.* 194: 121, 1939.
4. Selye, H.: On the Toxicity of Estrogen With Special Reference to Diethylstilbestrol, *Canad. M. A. J.* 41: 48, 1939.
5. MacBryde, C. M., Castrodale, D., Helwig, E. B., and Bierbaum, Olga.: Hepatic Changes Produced by Estrone, Estradiol and Diethylstilbestrol, *J. A. M. A.* 118: 1278, 1942.
6. Teague, R. S.: The Effect of Estrogens on the Microscopic Appearance of the Liver, *J. A. M. A.* 117: 1242, 1941. Teague, R. S.: Toxicology of the Synthetic Estrogen Diethylstilbestrol and Certain Related Compounds, *J. Pharmacol. & Exper. Therap.* 75: 145, 1942.
7. Tyslowitz, R., and Dingemans, E.: Effect of Large Doses of Estrogens on the Blood Picture of Dogs, *Endocrinology* 29: 817, 1941.
8. von Hamm, E., Hamann, T. E., Hardin, T. E., and Schoene, R. H.: Experimental Studies of the Activity and Toxicity of Stilbestrol, *Endocrinology* 28: 263, 1941.
9. Tyslowitz, R., and Hartman, C. G.: Influence of Large Doses of Estrogens on the Blood Picture of Rhesus Monkeys (*Macaca mulatta*), *Endocrinology* 29: 349, 1941.
10. Crafts, R. C.: The Effect of Endocrines on the Formed Elements of the Blood, *Endocrinology* 29: 606, 1941.
11. Good, C. A., Krumer, H., and Somogyi, M.: The Determination of Glycogen, *J. Biol. Chem.* 100: 483, 1933.
12. Elman, R., and Heifetz, C. J.: Experimental Hypoalbuminemia, *J. Exper. Med.* 73: 417, 1941.
13. Sobel, A., Yuska, H., and Cohen, J.: A Convenient Method of Determining Small Amounts of Ammonia and Other Bases by the Use of Boric Acid, *J. Biol. Chem.* 118: 443, 1937.
14. Graffia, Allan L., Marble, A., and Smith, R. M.: Note on Histological Estimation Versus Chemical Analysis of Liver Glycogen, *Anat. Rec.* 81: 495, 1941.
15. Janes, R. G., and Nelson, W. O.: Effect of Stilbestrol on Certain Phases of Carbohydrate Metabolism, *Proc. Soc. Exper. Biol. & Med.* 43: 340, 1940.
16. Griffiths, M., Marks, H. P., and Young, F. G.: Influence of Estrogens and Androgens on Glycogen Storage in the Fasting Rat, *Nature* 147: 359, 1941.

EFFECTS OF ETHER ANESTHESIA UPON TOTAL ERYTHROCYTE AND WHITE CELL COUNTS OF ADULT FEMALE RATS

ROGER C. CRAFTS, PH.D., BOSTON, MASS.

THE use of anesthesia in hematologic investigation has long been discouraged. Hemodilution, hemoconcentration, and contraction of the spleen have been described in texts of anesthesiology and pharmacology with the use of anesthetics of various types. Several recent papers have described a hemoconcentration in dogs with the use of ether anesthesia,¹⁻⁵ Barbour,⁶ however, found no hemoconcentration with ether in two rabbits, and Conley⁷ and Jareho⁸ reported no hemoconcentration with ether anesthesia in cats. Creskoff, Fitz-Hugh, and Farris⁹ reported that light ether anesthesia could be used in the rat without significantly altering the total erythrocyte count, though these authors do not quote any evidence for this statement. From the above data it seems that all species do not respond to ether anesthesia in a similar manner as far as plasma volume and the contraction of the spleen are concerned.

The present work on the effects of ether anesthesia upon the number of formed elements found in the blood of adult female rats was undertaken because (1) no detailed study has been reported on the effect of ether anesthesia on the total erythrocyte or white cell counts of the rat, and (2) since this animal is being used for the study of possible effects of endoerines upon the formed elements of the blood, it would be convenient to use a light ether anesthesia in obtaining blood samples.

METHODS

Adult female rats of the Long-Evans strain were employed for this study. They were 3 to 4 months of age and weighed 100 to 150 grams.

Blood was obtained by heart puncture, 0.1 c.c. of blood being removed for each sample. The blood was placed in a small vial containing a minute amount of powdered heparin to prevent clotting. Total erythrocyte and white cell counts were made with standard pipettes and an improved Levi-Hausser counting chamber. The same individual made all the counts.

RESULTS

In the first part of this study, 21 adult female rats were studied in an attempt to determine if light ether anesthesia for short periods of time alters the total erythrocyte or white blood cell counts (Table I). Each rat was lightly anesthetized and blood obtained by heart puncture. Less than two minutes elapsed between the time the rat was removed from the ether jar and the blood was obtained, the average time usually being one minute. This procedure, using ether anesthesia, was repeated three times at 10-day intervals. Total

From the Department of Anatomy, Boston University School of Medicine.
Received for publication, May 28, 1944.

erythrocyte and total white blood cell counts were done on the 63 samples of blood. The average total erythrocyte count was 8.38 million cells per cubic millimeter, with a mean deviation* of 0.46 million cells. The total white count was 7.92 thousand cells per cubic millimeter, with a mean deviation of 1.75 thousand cells. These figures compare favorably with a previous study of 62 unanesthetized adult female rats of this same strain¹⁰ in which the total erythrocyte count was 8.26 (± 0.68) million cells per cubic millimeter and the total white count was 8.88 (± 2.87) thousand cells per cubic millimeter.

Three more blood samples were then taken at 10-day intervals from each of these same 21 adult female rats using no anesthesia whatever, the same rats being used to eliminate individual differences. The average of the total erythrocyte counts of these 63 samples of blood was 8.39 million cells per cubic millimeter, with a mean deviation of 0.59 million cells; the white cell count 6.67 thousand cells per cubic millimeter, with a mean deviation of 1.65 thousand cells.

TABLE I

EFFECT OF LIGHT ETHER ANESTHESIA ON TOTAL ERYTHROCYTE AND WHITE CELL COUNTS OF ADULT FEMALE RATS

	LIGHT ETHER ANESTHESIA	NO ANESTHESIA
Rbc in millions	8.38 \pm 0.46*	8.39 \pm 0.59
Wbc in thousands	7.92 \pm 1.75	6.67 \pm 1.65

*Mean deviation.

These data indicate that the total erythrocyte count of the rat is not significantly altered by a short period of ether anesthesia. The total white cell count showed a rise from 6.67 thousand cells with no anesthesia to 7.92 thousand cells with anesthesia. This difference is not a significant one. The total white cell counts are quite variable under the best of conditions. Both of these average figures are within the normal range for total white cell counts in this strain of rats. The above data are summarized in Table I.

As all previous studies by other workers on the effects of ether anesthesia on the plasma volume and the contraction of the spleen utilized the ether for prolonged periods of time rather than one to two minutes, it was thought that a study of the effects of a longer period of ether anesthesia on the rat would be of interest. Accordingly, in the second part of this work, 10 adult female rats were anesthetized with ether and 0.1 c.c. of blood removed by heart puncture at 2, 6, 10, 20, 30, and 60-minute intervals after the rat was removed from the ether jar. Total erythrocyte and white cell counts were done on each sample of blood (Table II).

The average erythrocyte count for the 2-minute interval was 8.37 million cells per cubic millimeter. This agrees with the figures of 8.38 and 8.39 million cells obtained in the first part of this work. The total erythrocyte averages of 8.33, 8.20, 8.02, 7.96, and 7.84 million cells for the 6, 10, 20, 30, and 60-minute intervals respectively show a gradual decrease in the total number of erythrocytes per cubic millimeter. This decrease has been tested with various tests of

*As obtained by the formula: $E = \sqrt{\frac{\sum d^2}{N-1}}$

TABLE II

EFFECT OF PROLONGED ETHER ANESTHESIA ON TOTAL ERYTHROCYTE AND WHITE CELL COUNTS OF ADULT FEMALE RATS

	PERIOD OF ETHER ANESTHESIA IN MINUTES					
	2	6	10	20	30	60
Rbc in millions	8.37 ±0.51*	8.33 ±0.83	8.20 ±0.55	8.02 ±0.57	7.96 ±0.70	7.84 ±0.67
Wbc in thousands	6.45 ±1.60	7.32 ±2.11	6.41 ±1.45	6.42 ±2.05	6.64 ±1.30	6.94 ±2.40

*Mean deviation.

significance. It is not a significant change. The figure 7.84 million cells per cubic millimeter is within the standard deviation and any significant change should be at least twice the standard deviation. In addition, the above change is not biologically significant as many normal adult female rats show a count of 7.80 million cells per cubic millimeter. The total white count showed no significant change.

These data indicate that prolonged use of ether anesthesia, at least for sixty minutes, does not materially alter the total erythrocyte or total white cell counts in adult female rats (Table II).

As the time factor seemed to produce no pronounced effects, it was of interest to determine if the depth of anesthesia was the important factor. Fourteen adult female rats were very lightly anesthetized for fifteen minutes. Blood samples were then taken by heart puncture. After a 10-day interval the same rats were deeply anesthetized for the same length of time, fifteen minutes. Blood samples were again obtained by heart puncture and total erythrocyte counts made on each sample (Table III).

The total erythrocyte values of 7.84 million cells per cubic millimeter for the lightly anesthetized group, and 8.11 million cells for the deeply anesthetized group are both normal values. These data indicate that neither light nor deep ether anesthesia alters the total erythrocyte count of adult female rats.

TABLE III

EFFECTS OF LIGHT AND DEEP ETHER ANESTHESIA ON TOTAL ERYTHROCYTE COUNT OF ADULT FEMALE RATS

	LIGHT ETHER ANESTHESIA FOR 15 MINUTES	DEEP ETHER ANESTHESIA FOR 15 MINUTES
Rbc in millions	7.84 ± 0.80*	8.11 ± 0.64

*Mean deviation.

DISCUSSION

Ether anesthesia does not produce changes in the blood concentration in all animals. Although a hemoconcentration follows ether anesthesia in the dog, the rabbit and cat do not demonstrate any such change in blood concentration with the same anesthesia. The rat appears to belong in the group with the rabbit and the cat. The Long-Evans rats used in this study showed no significant change in the total erythrocyte or white cell counts when under the influence of ether anesthesia. Light anesthesia, deep anesthesia, anesthesia for short periods of time, and anesthesia for long periods of time produced no significant changes.

The source of this blood, the heart, should be emphasized. Whether peripheral blood would show any different results is problematical.

These data would seem to indicate that a light ether anesthesia for a 1- to 2-minute interval will in no way alter the number of formed elements in the blood of Long-Evans adult female rats when the heart is used as a source of blood. If anesthesia is used, heart punctures can be done repeatedly on rats with no signs of injury. The heart puncture following a short period of ether anesthesia will be utilized as a method of obtaining blood from rats in subsequent work in this laboratory.

SUMMARY

This work was an attempt to determine if ether anesthesia would alter the number of the formed elements in the blood of mature rats. Adult female rats of the Long-Evans strain were anesthetized for a 1- to 2-minute period, for a 60-minute period, anesthetized lightly, and anesthetized deeply. There was no significant change in either the total erythrocyte or the total white cell counts. Blood was obtained from the heart.

REFERENCES

1. Staader, H. J.: Studies in Anesthesia, Anoxemia, Anhydremia, and Eclampsia, With Certain Deductions Concerning the Treatment of Eclampsia, *Am. J. Obst. & Gynec.* 12: 633, 1926.
2. Searles, P. W., and Essex, H. E.: Changes in Blood in Course of Ether Anesthesia and Sodium Amytal Anesthesia, *Proc. Staff Meet., Mayo Clin.* 11: 481, 1930.
3. Bollman, J. L., Svirebely, J. L., and Mann, F. C.: Blood Concentration Influenced by Ether and Amytal Anesthesia, *Surgery* 4: 881, 1938.
4. McAllister, E. F.: Effect of Ether Anesthesia on Volume of Plasma and Extracellular Fluid, *Am. J. Physiol.* 124: 391, 1938.
5. Searles, P. W.: The Effects of Certain Anesthetics on the Blood, *J. A. M. A.* 113: 906, 1939.
6. Barbour, H. G.: Water Exchanges Due to Anesthetic Drugs, *Anesthesiology* 1: 121, 1940.
7. Conley, C. L.: The Effect of Ether Anesthesia on the Plasma Volume of Cats, *Am. J. Physiol.* 132: 796, 1941.
8. Jarcho, L. W.: The Effect of Nembutal-Ether Anesthesia Upon Blood Concentration, *Am. J. Physiol.* 138: 458, 1943.
9. Creskoff, A. J., Fitz-Hugh, T., and Farris, E. J.: The Rat in Laboratory Investigation, edited by J. Q. Griffith and E. J. Farris, Philadelphia, 1942, J. B. Lippincott Co., p. 351.
10. Crafts, R. C.: The Effect of Ether Anesthesia on the Formed Elements of the Blood. Part I. The Effects of Ether Anesthesia and Adrenalectomy on the Blood of the Adult Female Rat, *J. Biol. Chem.* 141: 941, 1942.

A VASOSPASTIC FACTOR IN THE SERUM OF A CASE OF RAYNAUD'S DISEASE WITH COLD AGGLUTINATION. EXPERIMENTS ON RABBITS

T. H. C. BENIANS, F.R.C.S. (ENG.),* LONDON, ENGLAND

THE case (Mrs. R.)† with which these experiments are concerned has already been published on account of the association of "Cold autohemagglutination with Raynaud's Disease" (Benians and Feasby, 1941). The relation of this condition to hemoglobinuria e frigore, in which Raynaud's syndrome is frequent, was pointed out and it was suggested that the vascular spasm might be allergic, i.e., an antigen-antibody stimulus leading to a vascular spasm, in this case a reversible process governed by the temperature of the blood. There are numerous substances which injected intravenously cause anaphylactoid death in the rabbit, and among these are cyto-toxic sera which are rich in heterophile antibodies. The serum of Mrs. R. contains agglutinins and lysins which act on rabbit's blood cells both in the cold and at body heat. The action of the antibodies on the rabbit blood cells is apparently not in itself the cause of the rapid anaphylactoid death which is due to spasm of the pulmonary arteries. It might, of course, be the case that this serum contains a toxic body which causes an arterial spasm having no relation to antigen-antibody reactions, but a mechanism of allergic type seems inherently probable. A few experiments in prophylactic treatment based on the idea of immunity reactions have been carried out and are recorded later.

Series 1.—Effects of "normal" serum. Rabbits are regarded as being exceptionally sensitive to intravenous injections of protein, so that some direct comparison is useful. Animals of various sizes as available were injected intravenously in a single injection with various amounts of "normal" human serum. Of these fourteen animals one died an anaphylactoid death, a second died a few hours after injection and probably as a result of it. Two were definitely shocked in the sense that they lay down and passed urine and feces but recovered after about fifteen minutes. The other ten suffered no obvious ill effect although three of them received 4 c.c. or more. Some of these sera taken at random from the laboratory bench evidently contained a toxic factor, the six rabbits, however, which were injected with serum from healthy "donors" showed no reaction at all, and this is in striking contrast with the fatal effects obtained with much smaller doses of the serum (Mrs. R.).

With the limited number of rabbits available one cannot be said to have fixed a minimal lethal dose but from Table II, and controls to individual experiments

*Pathologist to The North Middlesex County Hospital and The Prince of Wales's General Hospital, London, England.

†Under the care of Dr. R. Kempthorne, North Middlesex County Hospital.

Received for publication, Feb. 10, 1944.

TABLE I

WEIGHT OF ANIMAL	AMOUNT OF SERUM	SOURCE	RESULT
2.28 Kg.	1 c.c.	Donor*	Nil
2.28 Kg.	4½ c.c.	Donor	Nil
(Same rabbit 2 days later)			
1.14 Kg.	2 c.c.	Pooled sera from 2 W.R. cases	Nil
1.14 Kg.	3 c.c.	Pooled sera from 4 W.R. cases	Amphibicercoid death in 5 minutes
1.82 Kg.	2 c.c.	Pooled W.R. serum	Nil
0.79 Kg.	2 c.c.	Pooled sera	No effect that day but found dead next day No P.M.
1.14 Kg.	2 c.c.	Pooled sera	Nil
1.14 Kg.	3 c.c.	Pooled sera	Nil
1.59 Kg.	3 c.c.	Pooled sera	Uneasy ½ hour then recovered
2.28 Kg.	4½ c.c.	Donor	Nil
1.14 Kg.	2 c.c.	Donor	Nil
2.28 Kg.	2 c.c.	Old standing anemia now much improved	Uneasy ½ hour then recovered
1.36 Kg.	1 c.c.	Pooled sera from 3 donors	Nil
2.0 Kg.	3 c.c. plasma	Donor	Nil

*Different "donors" used on each occasion. Serum in all cases given intravenously at ordinary laboratory temperature.

TABLE II

EFFECTS OF SERUM (Mrs. R.) GIVEN INTRAVENOUSLY AT LABORATORY TEMPERATURE.

WEIGHT OF ANIMAL	DOSE	EFFECT
2.28 Kg.	5 c.c. fresh serum	Death in 1 minute
2.06 Kg.	5 c.c. fresh serum	Death in 1 minute
1.82 Kg.	1.5 c.c. fresh serum	Death in 1 minute
2.65 Kg.	1 c.c. fresh serum	Death in 9 minutes
2.88 Kg.	1 c.c. fresh serum	Moderate shock but survived
1.6 Kg.	1 c.c. fresh serum	Death in 5 minutes
1.0 Kg.	1 c.c. fresh serum	Death in 2½ minutes
1.0 Kg.	1 c.c. fresh serum	Death in 2 minutes

TABLE III

COMPARISON OF EFFECTS OF THE SAME SAMPLES OF SERUM (Mrs. R.) GIVEN AT LABORATORY TEMPERATURE AND WARMED TO 37° C.

1.59 Kg.	2.0 c.c.	10-day-old serum	Death in 10 minutes
1.48 Kg.	2.0 c.c.	Same serum warmed to 37° C. for 5 minutes and given at 37° C.	Shocked but recovered
2.28 Kg.	2.5 c.c.	6-day-old serum	Death in 2 minutes
2.28 Kg.	2.5 c.c.	Same serum warmed to 37° C. for 1 hour and given at 37° C.	Shocked but recovered
1.59 Kg.	1.0 c.c.	Fresh serum	Death in 2½ minutes
1.58 Kg.	1.0 c.c.	Same serum warmed to 37° C. for 15 minutes and given at 37° C.	Shocked but recovered
1.5 Kg.	0.5 c.c.	Fresh serum	Death in 5 minutes
1.43 Kg.	1.0 c.c.	Same serum warmed to 37° C. for 30 minutes and given at 37° C.	Severe shock for 1 hour but recovered
1.8 Kg.	1.0 c.c.	Fresh serum warmed to 37° C. for 30 minutes and given at 37° C.	Very severe shock; gradual recovery
2.3 Kg.	2.0 c.c.	Fresh serum warmed to 37° C. for 1 hour and given at 37° C.	Death in 5 minutes

given later, it appears probable that 1 c.c. of the fresh serum has a fatal effect on rabbits up to about 1.5 to 2 Kg. in weight.

In the experiments to be described later where a control test was carried out concurrently it is bracketed with its partner.

It is to be noted that although the warmed serum in equal or larger amount than the unwarmed has lost its lethal effect, except in one instance, it can still produce a considerable degree of shock. In the state here described as shock the animal lies down and passes urine and often feces, the ears are pale and cold, breathing is rapid, and the animal is limp to handle. There is no evidence of pain or paralysis. If put on its legs it will move off slowly but naturally to another part of the room. Recovery is gradual, usually in about half to one hour.

TABLE IV

A FEW TENTATIVE EXPERIMENTS SHOWING THE EFFECT OF TREATING THE FRESH SERUM (MRS. R.) IN VARIOUS WAYS

WEIGHT OF ANIMAL	DOSE OF SERUM INTRA- VENOUSLY	EFFECT
(1) <i>Heated to 56° C. for 30 minutes</i>		
1.5 Kg.	2.0 c.c.	Shocked but recovered
2.3 Kg.	3.0 c.c.	Slight shock only
1.5 Kg.	1.5 c.c.	No effect
(2) <i>Extracted by shaking with powdered activated charcoal, stood 30 minutes at laboratory temperature. Centrifuged clear</i>		
0.9 Kg.	2.0 c.c. extracted	Nil
1.6 Kg. (control)	1.0 c.c. untreated	Death in 5 minutes
1.5 Kg.	2.0 c.c. extracted	Nil
2.3 Kg.	3.0 c.c. extracted	Nil
2.5 Kg.	3.0 c.c. extracted	Nil
(3) <i>Extraction with washed packed human Group O corpuscles in equal bulk, shaken and kept at 4° C. 90 minutes then centrifuged clear in a cold bucket (marked hemolysis)</i>		
2 Kg.	3.0 c.c.	Nil
(4) <i>Globulins salted out from 5 c.c. dialysed and redissolved in 5 c.c. of saline</i>		
2.5 Kg.	5.0 c.c.	Death in 2 minutes
(5) <i>Effect of storage in refrigerator</i>		
1.7 Kg.	2.0 c.c. 5 weeks old sterile serum	Moderate shock, recovery
1.7 Kg. (control)	1.0 c.c. fresh serum	Death in 4 minutes

The experiments so far show definitely that the serum (Mrs. R.) contains a highly toxic factor for rabbits; the diminution of the lethal power of the serum when given warmed to 37° C. makes it almost certain that this factor is a "cold antibody."

Post-mortem examinations were carried out immediately after death on many of the cases and showed the general characters of anaphylactoid death in rabbits. No detailed study of the organs was made. Rapidly fatal cases usually showed as follows. Left heart tightly contracted and almost empty. Right heart and systemic veins, especially those of the abdomen and portal system, heavily distended with dark blood. A little clear free fluid in the abdomen in some cases. Pleurae clear. Lungs pale, limp and shrunken, only bleeding slightly at the cut surface. Stomach and intestine deeply congested. Kidneys

deep plum color on surface, on section the deeper cortical zone is relatively pale. Suprarenals show no abnormality. The blood shows some lysis, but agglutination was not a marked phenomenon. Coagulation of the blood in some fatal cases was delayed for upward of $1\frac{1}{2}$ hours in vitro, the clot was very fragile.

Microscopic sections of a few organs as follows. Cardiac septum anteriorly—coronary vessels empty but not contracted. Lung near hilum—tight contraction of the large arteries, most of which are nearly closed and empty, ruptures of the vessel walls are seen and many arteries lie in a wide sheath of extruded blood limited by the distended adventitial coat. The muscle fibers of the arteries in places show a very marked vacuolation with what appears to be complete destruction. Hemorrhage into alveoli was not noted to any considerable degree. There was some emphysema. Kidneys—the complex vascular mechanism has not been fully examined but in some cases marked contraction of the arcuate arteries is seen with severe vacuolation of muscle fibers of the media. This vacuolation is very much more marked than that seen in control rabbits killed by other methods, including a blow on the neck, coal gas, and chloroform, though it cannot be asserted that shock is entirely excluded in any of these forms of death. The cause of death is beyond doubt pulmonary artery spasm with obstruction to the blood flow.

PROTECTION AGAINST THE SERUM BY INJECTION OF LIPOID

Bearing in mind the relation of lipoids to the cell membrane in respect of permeability, their power of adsorption, the role of lecithin as hapten of the Forssman antigen, and especially its ability to function in the Wassermann reaction, it was decided to try its effect empirically as a protective agent. In the small number of experiments below it is shown to have had a protective effect in most instances. The suspensions of lecithin were made by floating a saturated alcoholic solution of ovoid lecithin on saline and gently rolling the tube to a uniform turbidity. Proportions of 10 per cent and 20 per cent of the alco-

TABLE V

WEIGHT OF ANIMAL	LECITHIN EMULSION	SERUM (MRS. R.)	EFFECT
2.3 Kg.	4.0 c.c. (10%)		No harmful effect
2.3 Kg.	4.0 c.c. (20%)		No harmful effect
2.7 Kg.	8.0 c.c. (20%)		No harmful effect
1.7 Kg.	7.0 c.c. (20%)		No harmful effect
1.75 Kg.	3.5 c.c. (10%)	2.5 c.c. (10-day-old serum)	No harmful effect
2.3 Kg.	5.0 c.c. (20%)	2.5 c.c. fresh serum	Shocked for $\frac{1}{2}$ hr. partial recovery; died following night
1.75 Kg.	5.0 c.c. (20%)	1.0 c.c. fresh serum	No effect
1.75 Kg. (control)	-	1.0 c.c. fresh serum	Death $2\frac{1}{2}$ minutes
1.6 Kg.	5.0 c.c. (10%)	1.0 c.c. fresh serum	Slight shock, recovery
1.6 Kg.	5.0 c.c. (10%)	1.0 c.c. fresh serum	Slight shock, recovery
1.6 Kg. (control)	-	0.5 c.c. fresh serum	Death 5 minutes
1.5 Kg.	4.0 c.c. (20%)	1.0 c.c. fresh serum	Death 5 minutes
1.5 Kg. (control)	-	0.5 c.c. fresh serum	Death 6 minutes
0.9 Kg.	4.0 c.c. (20%)	1.0 c.c. fresh serum	Death 4 minutes
1.0 Wg. (control)	-	0.5 c.c. fresh serum	Death 4 minutes

holie solution were used in experiments and the lecithin was injected intravenously just before injection of the serum.

The effect of lecithin itself was tested in a few preliminary experiments. The alcohol factor was not controlled. All injections were made at ordinary laboratory temperature. The lecithin used in most of the experiments was a pre-war jar of commercial ovolecithin (British drug houses) which had gone a deep brown color. A fresh pale preparation was used in a few of the experiments. Unfortunately the actual preparation used in any individual experiment was not recorded.

DISCUSSION

It may be that these experiments do not actually prove the point at issue which is that arterial spasm results directly from the action of "cold antibodies." They raise, however, a number of speculations which can be linked together.

The experiments relate to the serum of a patient which contains cold panhemagglutinins in high titer, and some lysins. This patient suffers from Raynaud's syndrome when cooled and, when really cold, exhibits a condition that may be called shock, with a feeling of constriction in the chest, shortness of breath, pale pinched facies, and blueness of the extremities. Her serum injected cold into rabbits causes lysis with a little agglutination of the blood and rapid death due to spasm of arteries. The spasm affects mainly the pulmonary arteries which may be ruptured, but is also seen in some of the renal arteries and in the vessels of the ears. The serum when given at body heat is seen to have lost much of its toxicity.

It seems likely that this violent effect in the rabbit has its counterpart in a mild and reversible form in the patient and that both could be regarded as allergic phenomena. Admitting that the experimental animal and the clinical case are widely different entities they have, in the experiments described, the essential point in common, i.e., that each shows arterial spasm when a "cold" panagglutinating antibody is circulating in the blood stream.

Antigen or antibody introduced into the blood stream and finding a suitable partner in the fixed tissues may unite with it and produce a reaction known as allergic or anaphylactic shock. Surface action, that is to say agglutination, precipitation, etc., is the obvious feature of antigen-antibody combination and the intense vacuolation seen in the muscle fibers of the media of the rabbit's pulmonary arteries bears some comparison with the dramatic clot-like agglutination which this serum causes in vitro when mixed with whole blood in the cold, and suggests an equally direct action on the muscle itself. It should be said that authorities, Topley and Wilson (1936) quoting various authors, regard the type of reaction shown in these injected rabbits as anaphylactoid and due to cytotoxic bodies in the serum, and this would be a feasible alternative explanation. For present purposes it is not necessary to adopt any specific nomenclature, provided the effects of this serum can be interpreted in terms of surface action.

There is some clinical support for a hypothesis that the action of auto-antibodies is related to vascular spasm. In a review of the hemoglobinurias Witts (1936) says "a feature that has not attracted the attention it deserves

is the occurrence of vascular disturbances which may threaten the life and comfort of the patient almost as seriously as the anemia or the suppression of urine." He also points out that shock is a prominent feature in intravascular hemolysis. Stats and Bullowa (1943) in an examination of all published cases of cold agglutination find twelve of them (roughly perhaps 30 per cent) showing functional vascular disease of the extremities. Their own case is one of only two in which the process has gone on to gangrene.

However attractive an allergic hypothesis might seem to be it is clear that the mere presence of high titer cold agglutinins does not necessarily lead to vasospasm. This effect so far seems to be limited to a class of case in which intravascular lysis is also taking place. An exception is a case of acrocyanosis recently reported by Helwig and Freis (1943) following atypical primary pneumonia; in this case there was no hemolysis and the authors attribute the stasis to the agglutination of the red cells. Stats and Bullowa had already adopted the same explanation in their case despite the fact that the obstruction was irreversible on warming.

Autoantibodies certainly exist in the blood plasma or they may develop or increase as the result of disease or experimental processes, thus Karady (1939) has apparently produced allergic shock in guinea pigs by exposure to both heat and cold, and seeks to explain some human allergies on this basis.

In the blood the iso-antibodies are always compatible with their accompanying cells, but in the "cold" antibodies we are concerned with factors which apparently are always incompatible, given adverse physiological conditions. Agglutinins and lysins of this type are known and the action of both is well seen in hemoglobinuria *e frigore*. It has to be borne in mind that, as ordinary immune bodies, these two factors are closely related and up to a point may be identical.

Cold hemagglutinins first demonstrated by Landsteiner (1903) are apparently a normal component of the plasma and at temperatures below 5° C. may be demonstrated in small amounts in most sera. When they are pathologically increased they usually function at higher temperatures, up to 25° C., although their power falls off rapidly as the temperature rises. Rosenthal and Corten (1937) in a study of these bodies conclude that the high titer cold agglutinins are not derived from an increase of normal "cold" antibodies for two reasons (1) that they can function at a higher temperature, and (2) that the reaction is massive and sudden. The point cannot be argued here but it seems probable that these are features of the strength of the factor rather than of its nature. It must be considered at present an open question whether the normal cold agglutinin is related to that which occurs during infections such as trypanosomiasis and atypical primary pneumonia, or whether either is the same as that which occurs in cold hemoglobinuria or, on rare occasions, in the hemolytic anemias.

Putting on one side the normal cold agglutinins it will be of interest to consider broadly and in two groups the bulk of the diseases in which a high titer serum is commonly found, because they appear at first sight to have no sort of connection with each other. The first group would include trypanosomiasis and atypical primary pneumonia. The former is essentially a protozoal

infection of the blood stream and in many aspects its morbid anatomy is akin to syphilis. As well as cold agglutinins a pseudopositive Wassermann reaction occurs in this disease. The latter disease, though not yet well defined, is believed to be due to a virus infection. In this condition Drew, et al. (1943) state that a positive Wassermann reaction is not uncommon. Cold agglutinins were demonstrated by Peterson (1943) and used as a diagnostic measure by Turner, et al. (1943) who have shown that these agglutinins show a rise and fall in the manner of other immune bodies.

The second group includes cold hemoglobinuria, in which Raynaud's syndrome is a frequent and severe symptom and cold agglutinins and lysins are constant; and secondly those cases of cold agglutination associated with hemolytic anemia or some other form of blood disease. In the first of these (cold hemoglobinuria) a positive Wassermann reaction is so frequently present as to lead to its being regarded as a syphilitic disease, which of course it may be, although a diagnosis of syphilis based only on the Wassermann reaction may be fallacious in such cases owing to the presence of high titer heterophilic antibodies in the serum. In the hemolytic anemia type of case a positive Wassermann reaction is also frequent. Apart from the cold agglutinins the Wassermann body thus forms a tenuous link binding these diverse diseases together.

The Wassermann body is regarded as a product of autoimmunization to damaged tissues and, since syphilis is essentially a vascular disease, it is not a mere coincidence that the other factor functioning in the complement fixation test should be an extract of vascular-system tissue, i.e., heart muscle. It is to be noted that lecithin can also function in this reaction as an alternative to heart muscle.

We have then some reason for regarding the high titer cold agglutinin as an immune body arising in many instances parallel with the Wassermann body and perhaps mainly as a reaction to blood vessel damage. In its turn it could, theoretically, affect the media of the arteries (for this is the tissue mainly damaged in syphilis) and so lead to spasm, although admittedly the Wassermann body does not in general react so itself. The easiest explanation of why its spastic action is mainly seen in the cold would be to presume that it was built up on an autochthonous prototype. Turner, et al., while taking many of the above-mentioned factors into consideration conclude that the cold agglutinin arising in atypical primary pneumonia may be either a direct antibody to the unknown infecting agent, or a response to damaged tissues perhaps of the respiratory tract.

Seeing that cold agglutinins are a normal constituent of the blood plasma it might not be out of place to consider finally whether they have, or have not, a teleological value to the organism. Their function, if any, would lie in stopping the blood flow in an excessively cooled part at the periphery and so preventing further loss of heat in the blood still circulating. Vasomotor control through the nervous system is a relatively late arrival in the course of evolution and it may be that some controlling mechanism of humoral type preceded it.

The first response of superficial vessels to cold is a local one as Lewis (1941) has shown and the contraction affects vessels of all types, but no doubt

the arterial types most, on account of their uniscler element. It is in this mechanism of contraction that a physiologic factor in the cooled plasma might come into play. This, however, is a mere supposition, in any case it is probably a truism (altering Osler's dictum) that man is as cold as his arteries.

SUMMARY

A serum containing high titer cold antibodies, and derived from a case of Raynaud's disease, is shown to cause fatal pulmonary artery spasm in rabbits when given, cold, intravenously. This effect is mitigated by giving the serum warm. It is suggested that these cold antibodies have a direct effect, probably of an allergic type, on arterial musculature both in the experimental animal and the clinical case. It is further suggested that the frequent association of cold antibodies with a Wassermann-like body point to an origin of the former from diseased vascular structures and this again would help to explain their action on both blood cells and vessels. A possible function of cold antibodies in normal vascular control is mentioned, these may or may not be the basis or prototype of the high titer cold antibodies. Preliminary experiments in protection against the cold antibodies by the intravenous injection of lipoids have been carried out with some success.

REFERENCES

- Benians, T. H. C., and Fearby, W. R.: Raynaud's Syndrome With Spontaneous Cold Haemagglutination, *Lancet* 2: 479-480, 1941.
- Drew, W. R. M., Samuel, E., and Ball, M.: Primary Atypical Pneumonia, *Lancet* 1: 761-765, June, 1943.
- Helwig, F. C., and Freis, E. D.: Cold Autohaemagglutinins Following Atypical Pneumonia Producing the Clinical Picture of Acrocyanosis, *J. A. M. A.* 123: 10, 626.
- Karady, S.: Role of Auto-Antigens in Pathogenesis of Physical Allergy, *J. Immunology* 37: 457-461, 1939.
- Landsteiner: Ueber Beziehungen zwischen dem Blutserum und den Körperzellen, München. med. Wchnschr. 50: 1812, 1903.
- Lewis, T.: Observations on Some Normal and Injurious Effects of Cold Upon Skin and Underlying Tissues; Reactions to Cold and Injury of Normal Skin (Holme) Lecture, *Brit. Med. J.* 2: 795-797, 1941.
- Peterson, O. L., Ham, T. H., and Finland, M.: Cold Agglutinins (Autohaemagglutinins) in Primary Atypical Pneumonias, *Science* 97: 167, 1943.
- Rosenthal and Corten: Ueber das Phänomen der Autohaemagglutination und über die Eigenschaften der Kaltehaemagglutinine, *Folia haemat.* Fol. Haematol. 58: 64-90, 1937.
- Stats, D., and Bullock, J. G. M.: Cold Haemagglutinins With Symmetrical Gangrene of the Tips of the Extremities, *Arch. Int. Med.* 72: 4, 506, 1943.
- Topley and Wilson: Principles of Bacteriology, London, 2nd. Ed., 896, 1936.
- Turner, J. C., Nisnewitz, S., Jackson, E. B., and Berney, R.: Relation of Cold Agglutinins to Atypical Pneumonia, *Lancet* 1: 765-769, June, 1943.
- Witts, L. J.: Paroxysmal Haemoglobinurias, *Lancet* 2: 115-120, 1936.

THE COLD-SUSCEPTIBLE GLOBULIN FRACTION OF PATHOLOGIC SERA

E. WERTHEIMER, M.D., AND L. STEIN, M.D., JERUSALEM, PALESTINE

IN AN earlier paper it was shown that sera of dogs and human beings infected with kala-azar contain a protein fraction closely related to, but even more labile than, euglobulin.³ An outstanding characteristic of this pathologic fraction is its deposition as a flocculent precipitate in the cold and its tendency to revert to a dissolved state when the temperature is suitably raised (37° C.). It was designated the Cold Fraction (C.F.) and defined as that protein portion of serum which precipitates when the serum stands for twenty-four hours at from 7 to 11° C. The present investigation deals further with the significance of the C.F.

METHODS

Findings reported in our earlier paper formed the basis of the present. It was found necessary in many cases to determine the amount of the C.F. after three days rather than after twenty-four hours of standing in the refrigerator, since precipitation in the shorter interval was often incomplete. A slight serum turbidity is often observed in pathologic cases. It has not been connected diagnostically with any particular condition. Successive grades of abundance of C.F. in serum were designated as follows: 1, turbidity marked but no precipitate; 2, light precipitate; 3, flocculent precipitate; 4, marked precipitate easily estimable by quantitative methods; 5, precipitate of a different nature present as a layer on the surface.

Essential features of the reversion of precipitation have been described in an earlier paper. Chemical methods were as described in our earlier paper.

Dilution fraction (D.F.) designates that fraction of globulin which is particularly sensitive to absence of electrolytes and which precipitates five minutes after dilution of 1 part serum with 30 parts of distilled water.

RESULTS

C.F. in Human Beings Infected With Kala-Azar.—Five patients with kala-azar were examined. Two had mild infections and could be tested on one occasion only. In both of these C.F. grade 3 was recorded. The results obtained in the three other patients with kala-azar will be reported in detail because they are in certain respects peculiar (Table I).

Case S. represents an infection with kala-azar derived from Turkestan. The disease at examination was less than of five weeks' duration. The condition was relatively mild and readily amenable to treatment. It is noteworthy that although the formol-gel reaction was found negative, the D.F. only a trace, and

From the Laboratory of Pathological Physiology, the Hebrew University.

TABLE I
 HUMAN BEINGS WITH KALA-AZAR

PATIENT	DATE	TOTAL PROTEIN	ALBUMIN	GLOBULIN	EUGLOBULIN	C.F.	D.F.	FORMOL-GEL	REMARKS
S.	6/10/43	8.36	3.78	4.58	Slight increase	1.56%	Trace	0	Treated with stilbene
	6/21/43	6.70	3.60	3.10	0.29	3	Trace	0	Treated with stilbene
	7/ 6/43	7.50	4.42	3.08	Slight increase	Trace	0	0	Clinical improvement
	8/11/43	6.65	4.70	1.95	Normal	0	0	0	Clinically normal
P.	6/ 7/42	10.00	-	-	4.20	3	+	Instantaneous +	Treated with stilbene
	7/ 2/42	10.80	2.88	7.92	4.40	3	1.86%	Instantaneous +	Treated with stilbene
	7/31/42	12.00	1.30	11.7	1.25	3	2.37%	Instantaneous +	Treated with stilbene
	8/ 6/42	10.80	-	-	Increase	0.27%	+	Instantaneous +	Treated with stilbene
	8/13/42	10.44	3.24	7.20	4.31	0.22%	1.16%	Instantaneous +	Clinically normal
	9/ 7/42	8.52	4.44	4.08	Heavy increase	0.24%	++	Instantaneous +	Clinically normal
	10/11/42	10.05	3.93	6.12	Increase	0.30%	++	Instantaneous +	Clinically normal
	1/21/43	8.72	5.50	3.22	Normal	3	++	++ (After several hours)	Clinically normal
	2/ 3/42	9.23	2.70	6.53	Heavy increase	3-4	++	++ (After 2 hr.)	
	2/24/42	8.04	3.43	4.61	2.48	3-4	+	++ (After 3 1/2 hr.)	
G.	4/14/42	7.18	3.44	3.74	Increase	3-4	+	++ (After 6 hr.)	
	5/ 1/42	7.72	3.95	3.77	Increase	3-4	(+)	++ (After 2 hr.)	
	7/30/42	6.09	4.21	1.88	Slight increase	3	Trace	0	Clinically normal
	9/25/42	5.71	3.24	2.47	Normal	3	0	0	Clinically normal
	3/ 1/43	7.44	4.90	2.54	Slight increase	3	Trace	0	Clinically normal

euglobulin increased but slightly, the C.F. was highly abundant. It disappeared rapidly together with the recession of the total globulin under treatment. Whether, as this suggests, presence of C.F. is of diagnostic value in early stages of kala-azar can be decided only on the basis of more extensive data. Case S. shows clearly that C.F. is not identical with the protein conditioning the formol-gel reaction nor with that which is precipitated by distilled water (D.F.). The property of cold precipitability seems to be not an acquired property of known protein fractions but the characteristic of a specific pathologic fraction.

Patient P., in contrast to Patient S., had a marked increase in blood globulin extending to the different fractions included in this term. The increase was marked even after the patient had achieved complete clinical recovery. An analysis carried out five months after recovery revealed a return of the total globulin content to practically normal and a negative formol-gel reaction but positive D.F. and C.F. tests.

Patient G. showed a similar trend. He had a clinical recovery; the serum protein value was at normal level; the formol-gel reaction, negative; the D.F., nil or traces; the euglobulin, normal or slightly increased. Nevertheless a C.F. was still clearly demonstrable six months after clinical recovery, although, of course, it was smaller after this time than during the heyday of the disease.

The clinical improvement is characterized by rise of albumin, fall of globulin and, particularly, euglobulin, and decrease of the pathologic fractions. The C.F. in particular may persist long after clinical recovery is considered to be complete. The question thus arises whether a patient whose serum is found to contain easily demonstrable pathologic protein fractions can be nevertheless regarded as completely cured. It is conceivable that the blood characteristics of such a patient are due to a condition of hyperstimulation of the reticulo-endothelial system.

Of thirteen cases of *Leishmania cutis* (Jericho rose) which came under examination, only 1 chronic case gave a positive C.F. test; it was graded 2.

C.F. in Dogs Infected With Kala-Azar.—Three further cases were examined—a mild, a medium, and a severe. Table II shows the results of the blood examinations as obtained when the dogs first came under examination.

TABLE II

CASE	TOTAL PROTEIN	ALBUMIN	GLOBULIN	EUGLOBULIN	C.F.	D.F.	FORMOL-GEL
1	6.69	2.44	4.25	0.69	0.32%	-	+ after 1 min.
2	10.06	2.63	7.43	2.45	0.37%	0.68%	+ after 1½ min.
3	12.38	2.00	10.38	5.47	3-4	1.1 %	+ instantaneously

TABLE III

DIAGNOSIS	TOTAL PROTEIN	ALBUMIN	GLOBULIN	EUGLOBULIN	C.F.	D.F.	FORMOL-GEL	REMARKS
Sepsis	6.23	3.47	2.76	Slight increase	3-4	+	-	Died
Malaria (chronic)	7.56	3.67	3.89	Increase	0.26%	+	-	Cholesterol, 160 mg. %
Malaria (chronic)	6.25	3.34	2.91	Increase	4	0	0	
Lepa	6.56	5.00	1.56	Normal	3-4	0	0	

The tabulated data corroborate that (except for the C.F. which is independent of all other fractions and seemingly so also of other elements making up the clinical picture) the tested globulin fractions parallel one another.

Other than in kala-azar, C.F. has been found positive in a few other cases of chronic infections. Table III describes the outstanding instances. The data in Table III also confirm that the C.F. is independent of the other globulin fractions.

ENDOCARDITIS LENTA

The condition of endocarditis lenta was selected for a special study since it seemed possible that C.F. in this condition might prove to be a character of diagnostic value. It should be emphasized in advance that the C.F. in cases of endocarditis lenta is meager as compared to the C.F. usually found in kala-azar and is only in exceptional circumstances accessible to quantitative estimation. It should be noted further that the precipitation is relatively sluggish and generally complete only after from three to four days. At 37° C. the sediment can, in all cases, be brought again into solution.

The impression is obtained that the C.F. increases if the disease becomes aggravated. Sulfanilamide in serum can inhibit or prevent the appearance of the C.F. As a comparison reference, eighteen sera from cases of endocarditis rheumatica were examined. Only one of them yielded a positive C.F. test, the precipitate obtained in this case being of a plastic irreversible type. In all the examined sera correlation between C.F., D.F., englobulin, and formol-gel reaction was not in evidence. A summary of the findings is presented in Table IV.

TABLE IV
ENDOCARDITIS LENTA

DATE	PATIENT	TOTAL PROTEIN	ALBU- MIN	GLOBU- LIN	EUOLORU- LIN	C.F.	D.F.	FORMOL-GEL
7/20	H.	6.3	3.4	2.9	1.5	1	0	After 1 hr. +
7/23		5.9	3.0	2.9	0.8	1	0	After 2½ hr. +
9/11		7.95	2.6	5.0	2.1	3	0	
9/18	Fin.	6.72	3.50	3.22	0.57	0	+	After ¼ hr. +
10/19		7.82	3.10	4.72	1.62	3	+	After 1½ hr. +
2/16	Lib.	8.98	3.11	5.87	3.4	2	+	
3/5		7.50	3.5	4.0	1.3	1-2	Trace	After 1 hr. +
3/30		6.8			1.1	3		
2/17	Saf.	9.1	4.6	4.5	1.7	3	0	
2/19		9.31	5.0	4.3	1.2	2	0	0
4/20	St.	8.16	3.3	4.9	Increase	3	+	
4/27		7.38			0.5	3	+	
1/12	Lech.	8.23	4.33	3.90	Increase	1.8%	+	Instantaneous +
2/12		8.55			Increase	2.5%	+	Instantaneous +
6/ 1	Brs.				Normal	3	+	0
6/ 8	Gl.	7.45	3.44	4.01	Increase	3	0	0
6/21					Increase	3	0	0

Although the amount of material examined so far is small, the possibility seems to be definitely indicated that presence of C.F. can have diagnostic significance in endocarditis lenta. Conclusive decision on this point must await the accumulation of more extensive data.

COLD PRECIPITATES IN SPECIAL SERA FROM CASES OF OTHER DISEASES (TABLE V)

In nephroses especially, serum samples frequently, although not always, become turbid in the cold, and after some time deposit a precipitate in the form of a ring near the surface. This can be redissolved at 37° C. rapidly and reappears if the serum is again cooled. Analysis of the fraction showed that it consisted largely of cholesterol and, in small part, of protein. The cholesterol level of the serum is not, however, determinant of the appearance of this cold fraction. In the so-called nephroses of pregnancy, positive C.F. tests have not been encountered.

It is pertinent to note that C.F. tests similar to those of nephroses have been recorded in three cases of morbus Gaucher and in one case of Niemann-Pick disease. Finally it may be mentioned that cold fractions, generally of an irreversible type, are encountered in the final stage of chronic uremia, in severe cirrhotic alterations of the liver, and also in cases of acute liver atrophy. Investigations designed to elucidate the nature of these cold fractions have been put under way. In all cases of this category, englobulin was normal; the D.F., negative; and the formol-gel reaction, 0.

TABLE V
COLD FRACTION IN OTHER DISEASES

PA-TIENT	DIAGNOSIS	TOTAL PROTEIN	ALBU-MIN	GLOBU-LIN	C.F.	UREA	URIC ACID	CHOLESTEROL
Ko.	Amyloid nephrosis; osteomyelitis	5.78	2.66	3.12	5	56	-	242
Sp. 1/18	Chronic nephritis and nephrosis	3.81	1.23	2.58	5	28	3.0	628
3/16	Chronic nephritis and nephrosis	4.46	2.55	1.91	3	52	4.4	1,000
5/4	Chronic nephritis and nephrosis	-	-	-	5	70	-	955
R.	Nephrosis; uremia	4.84	3.15	1.69	5	105	8.1	356
D.	Nephrosis 7/22	5.33	3.12	2.21	3	35	-	660
	8/7	4.57	1.44	3.33	5	32	-	635
B.	Chronic nephritis and nephrosis	5.13	2.38	2.75	3	143	-	386
L.	Nephrosis	4.95	3.10	1.85	1	-	-	515
F.	M. Gaucher	6.66	4.17	2.49	5	-	-	121
Z.	M. Gaucher	7.50	5.32	2.18	3	-	-	90
					(Irreversible)			
X.	M. Gaucher	-	-	-	5			125
W.	M. Niemann-Pick	6.54	3.48	3.06	5	-	-	219
R.	Chronic nephritis; uremia	7.40	3.60	3.80	4	315	10.0	(Xantho-protein ++)
					(Irreversible)			
S.	Hypertension; uremia	7.23	4.40	2.83	4	200	6.8	(Xantho-protein ++)
					(Irreversible)			
G.	Chronic nephritis; uremia	-	-	-	3	136	6.3	(Xantho-protein +++)
					(Irreversible)			
L.	Cirrhosis, hepatic; hemachromatosis	6.08	3.17	2.91	3	21	-	285
					(Irreversible)			

HYPERGLOBULINEMIA AND PATHOLOGIC SERUM PROTEIN FRACTIONS IN PROTEIN HUNGER AND BLOOD-LOSS (TABLE VI)

Numerous investigations have revealed that in chronic protein hunger, blood loss, or plasmapheresis, either separately or combined, serum globulins

TABLE VI

DATE	TOTAL PROTEIN	ALBU- MIN	GLOBU- LIN	ERGLO- BULIN	C.F.	D.F.	FORMOL- GEL	BLOOD WITH- DRAWN (C.C.)	HEMAT- OCRIT (HB.)	ERYTH- RO- CYTES MIL- LIONS
<i>Dog L. (26 kg.)</i>										
6/23	10.06	2.65	7.43	2.45	0.37	0.68	(1½ min.)	-	58 27	3.7
7/26	8.79	1.73	7.08	1.68	+ (None de- termi- nable)	0.86	-	-	55 29	4.08
8/ 4	10.94	1.71	9.23	4.69	0.50	1.13	+ (15 sec.)	600	58 28	4.25
8/ 5	9.56	1.05	8.51	5.06	1.13	0.56	-	75	43 26	3.11
8/ 6	10.63	1.32	9.31	5.88	2.50	0.69	Instanta- neous	70	50 24	3.35
9/10	10.00	0.94	9.12	5.00	0.73	0.23	-	-	-	-
<i>Dog B. (21 kg.)</i>										
3/24	12.38	2.0	10.38	5.47	+ (None de- termi- nable)	+ (None de- termi- nable) (1.1 5/27)	Instanta- neous	-	78 30	6.0
6/12	11.63	1.68	10.45	4.63	+ (None de- termi- nable)	2.37	Instanta- neous	220	65 26	5.1
6/26	11.63	1.63	10.00	5.88	1.00	1.00	Instanta- neous	540	43 21	3.35
7/ 6	11.13	1.73	9.38	5.00	0.38	3.38	Instanta- neous	590	38 20	2.19
7/13*	10.25	2.25	8.00	2.87	1.45	1.37	Instanta- neous	120	34	2.75
7/15	9.38	2.63	6.75	2.00	+ (None de- termi- nable)	2.63	Instanta- neous	-	-	2.97
7/17	9.56	2.16	6.40	5.56	+ (None de- termi- nable)	0.25	Instanta- neous	60	55 29	2.78

*Meat diet from 7/13, condition—very poor.

are sustained at the normal level although serum albumins are decreased.¹ It appeared pertinent in view of our findings in kala-azar to establish whether the abnormally increased serum globulin fractions of this condition are affected by protein deficiency in the diet and by blood loss.

Two dogs (kala-azar) with marked hyperglobulinemia were selected for the purpose of these experiments and maintained on a protein-low diet.⁴ At intervals of from two to three days blood was let from the animals in varying quantities.

Dog L. in the first stages of the experiment was reinfused with a suspension in saline of the removed erythrocytes (plasmapheresis). In a later stage reinfusion was omitted and the protein deficiency condition thus further enhanced. This treatment raises the need of hemoglobin neogenesis, which, according to Robscheit-Robbins and associates,² takes precedence over formation of other serum proteins. In the experiment with Dog L. it is particularly evident that, despite marked protein deficiency in the diet and serious loss of blood, the total globulin, euglobulin, and the pathologic C.F. and D.F. continue their upward trend. In Dog B. the globulin level again was maintained and the pathologic fractions showed some increase. The experiments show that the greatly enhanced levels of globulin and pathologic protein are maintained despite chronic protein hunger combined with severe blood loss (Table VI).

It is clear that the production of this protein material is exclusively effected at the expense of endogenous materials and is entirely independent of dietary protein supply and of the protein reserves. On feeding of protein (meat) after prolonged protein hunger and after numerous blood lettings, the globulin values and the pathologic protein fractions of Dog B. were not varied, whereas the albumin values showed an immediate increase.

DISCUSSION

The data available suggest that cold labile globulin (or globulins) forms a pathologic fraction independent from other globulins and not necessarily parallel in trend with the latter. The salient characteristic of this fraction is its marked lability, a circumstance which renders electrophoretic investigation difficult or impossible. C.F. occurs most uniformly and in greatest abundance in kala-azar. Its possible diagnostic value in this condition, provided present experience is confirmed on a larger scale of material, has been indicated. The C.F. may also occur in other highly chronic infections, such as malaria and tuberculosis. Particular interest attaches to its occurrence in endocarditis lenta. It has been suggested that in the latter condition the C.F., although not abundant, may prove to be of diagnostic value. The form of distribution of the C.F. suggests that in chronic infections, which evoke strong defensive reactions, the sustained stimulation of the globulin-forming apparatus leads not only to increased formation of euglobulin, but also to the release into the blood of a serum-incompatible, particularly labile, globulin fraction. A C.F. of a very different nature is encountered in nephroses. In these conditions the C.F. consists largely of cholesterol but is nevertheless independent of the general blood cholesterol level. In conditions of uremia and liver cirrhosis in the final stage, an irreversibly precipitable C.F. is frequently encountered. The nature of this C.F. is still completely unknown. The experiments with dogs infected with kala-azar show that the enhanced production of globulin and the formation of pathologic globulin fractions in this condition is not dependent either on dietary supply of protein or on the protein reserves of the organism.

SUMMARY

The cold-precipitable protein found in patients with kala-azar (human and dog) forms an independent pathologic fraction separate in behavior and formation from other investigated globulins.

The C.F. can be demonstrated in the serum long after the patients are clinically recovered.

The manifold increase in the level of total globulin, euglobulin, the C.F., and the other pathologic fractions of patients with kala-azar are maintained despite chronic protein hunger and extensive blood loss. The production of the increased protein fractions is therefore independent of the dietary protein supply and of the body protein reserves. The presence of C.F. in cases of endocarditis lenta has been demonstrated and its possible diagnostic significance has been pointed out.

In severe lipid nephroses, final phase of chronic uremia, and in severe cirrhotic alterations of the liver, C.F. of a peculiar nature occurs in the serum.

We are indebted to Prof. S. Adler of the Parasitological Department and to Dr. Tschernomoritz for their kind and generous help.

REFERENCES

1. Pommerenke, W. T., Slavin, H. D., Kariher, D. H., and Whipple, G. H.: Blood Plasma Protein Regeneration Controlled by Diet, *J. Exper. Med.* 61: 261, 1935.
2. Rolschait-Robbins, F. S., Miller, L. L., and Whipple, G. H.: Hemoglobin and Plasma Protein, *J. Exper. Med.* 77: 373, 1943.
3. Stein, L., and Wertheimer, E.: A New Fraction of Cold-Susceptible Protein in Blood of Dogs Infected With Kala-Azar, *Ann. Trop. Med.* 36: 17, 1942; Proteins Susceptible to Cold in Pathological Sera, *Nature* 149: 528, 1942.
4. Weech, A. A., Goetsch, M. D., and Reeves, E. B.: Nutritional Edema in the Dog, *J. Exper. Med.* 61: 299, 1935.

LABORATORY METHODS

GENERAL

GENTIAN VIOLET-BLOOD AGAR PLATES USED IN AEROBIC AND ANAEROBIC CULTURES OF WOUNDS

LIDA F. HOLMES, PH.D., AND MARY E. WILSON, A.B., PHILADELPHIA, PA.

GENTIAN violet incorporated into media to produce selectivity of bacterial growth has been used for many purposes. Churchman¹ separated anthrax from a gram-negative rod and isolated each organism in pure culture by employing gentian violet and acid-fuchsin. Farley^{2, 3} has used gentian violet as an acid in isolation of pathogenic molds. That gentian violet assists in isolation of the influenza bacillus was shown by Bernstein and Lowe.⁴ This dye has also been used in bacterial counts of air, being employed in such studies by Thomas,⁵ Thomas and Van den Ende,⁶ Bourdillon, Lidwell and Thomas.⁷ False presumptive tests for *B. coli* in water and milk analyses occur less frequently if gentian violet is incorporated into lactose broth than when lactose broth is used without an inhibiting agent. This work was done by Hall and Ellefson,^{8, 9} who reported that the percentage of samples positive for *B. coli* was not lowered.

Petroff's medium for culture of the tubercle bacillus employs gentian violet in a concentration of 1:10,000 to discourage growth of molds and other common contaminants. The fact that acid-fast organisms, although gram-positive, are highly resistant to gentian violet, was first noted by Churchman¹⁰ and has been elucidated by the work of Stearn and Stearn¹¹ who found that tubercle bacilli decompose gentian violet.

The dye, incorporated in blood agar plates, was used in a wound study reported by Francis,¹² who found in 300 swabs from burns and wounds inoculated in parallel on plain blood agar and gentian violet-blood agar that 32.7 per cent were positive for beta hemolytic streptococci when the dye was present, whereas, only 22.7 per cent were positive otherwise.

Gentian violet is known to be of definite aid in purifying anaerobes when their cultures are contaminated with *B. subtilis*, and has been used by Hall¹³ routinely as an aid in isolation of anaerobes.¹⁴ No reports, however, have been made of the numbers and types of anaerobes isolated in a series of cultures where gentian violet medium was compared with a dye-free medium.

From the Harrison Department of Surgical Research, Schools of Medicine, University of Pennsylvania, Philadelphia; and the City Hospital of Akron, Akron, Ohio.

The work described in this report was done under a contract recommended by the Committee on Medical Research between the Office of Scientific Research and Development and the University of Pennsylvania, and is part of the Contaminated Wound Project of the Subcommittee on Surgical Infections.

Received for publication, June 16, 1944.

The present work concerns 266 duplicate platings of either swabs or pieces of débrided tissue from wounds and burns cultured directly, or from platings of meat tubes previously inoculated with such swabs or tissue. The duplicate platings were made on 5 per cent blood plates of Difco's beef heart infusion agar with and without gentian violet in a final concentration of 1:500,000. About half of the platings were aerobic, the rest anaerobic. Identification of

TABLE I

ORGANISM	BLOOD AGAR PLATES ONLY	GENTIAN VIOLET- BLOOD AGAR PLATES ONLY	APPEARED ON BOTH
<i>Bacillus subtilis</i> and other gram- positive aerobic sporeformers	44	1	22
<i>Bacterium aerogenes</i>	1		3
<i>Bacterium alkalescens</i>	1	11	7
<i>Bacterium coli</i>		1	12
<i>Bacteroides melaninogenicus</i>			2
<i>Bacteroides</i> (other than <i>melaninogenicus</i>)		2	1
<i>Clostridium</i> species			
<i>Cl. bifementans</i>	1	1	6
<i>Cl. botulinum</i> Type A			1
<i>Cl. capitovialis</i>		3	
<i>Cl. cochlearium</i>	1	1	3
<i>Cl. histolyticum</i>		1	
<i>Cl. non-fermentans</i>	1		
<i>Cl. paraputrificum</i>		1	
<i>Cl. putrificum</i>		1	
<i>Cl. sporogenes</i>	3	2	6
<i>Cl. subterminalis</i>		1	
<i>Cl. tertium</i>	1	1	2
<i>Cl. welchii</i>	1	4	8
Unidentified	3	5	6
New species	1		
<i>Corynebacterium</i> (aerobic)	14		7
<i>Corynebacterium</i> (anaerobic)	2	1	1
<i>Escherichia enterica</i>	1		
<i>Flavobacterium</i> group		3	3
<i>M. candidans</i>	1		
<i>M. candidus</i>			2
<i>M. epidermidis</i>	2		
<i>M. nitrificans</i>			2
<i>M. ochraceus</i>			1
<i>M. titragenus</i>	3		
<i>N. catarrhalis</i>	1	1	
<i>Proteus</i> group			5
<i>Ps. pyocyaneus</i>			4
<i>Sarcina lutea</i>	1		
<i>Staph. albus</i>	1		5
<i>Staph. anaerobic</i>	7	12	21
<i>Staph. aureus</i>	89	6	30
<i>Staph. epidermidis</i>	6		3
<i>Strep. aerobic nonhemolytic</i>	8	13	46
<i>Strep. aerobic alpha hemolytic</i>		11	12
<i>Strep. aerobic beta hemolytic</i>	3	8	18
<i>Strep. anaerobic nonhemolytic</i>			3
<i>Peillonella garzogenes</i>		1	
Totals	197	92	244

SUMMARY

	AEROBES	ANAEROBES
Isolated on blood agar plates only	176	21
Isolated on gentian violet-blood agar plates only	55	37
Isolated on both	182	62

LABORATORY METHODS

GENERAL

GENTIAN VIOLET-BLOOD AGAR PLATES USED IN AEROBIC AND ANAEROBIC CULTURES OF WOUNDS

LIDA F. HOLMES, PH.D., AND MARY E. WILSON, A.B., PHILADELPHIA, PA.

GENTIAN violet incorporated into media to produce selectivity of bacterial growth has been used for many purposes. Churchman¹ separated anthrax from a gram-negative rod and isolated each organism in pure culture by employing gentian violet and acid-fuchsin. Farley^{2, 3} has used gentian violet as an acid in isolation of pathogenic molds. That gentian violet assists in isolation of the influenza bacillus was shown by Bernstein and Lowe.⁴ This dye has also been used in bacterial counts of air, being employed in such studies by Thomas,⁵ Thomas and Van den Ende,⁶ Bourdillon, Lidwell and Thomas.⁷ False presumptive tests for *B. coli* in water and milk analyses occur less frequently if gentian violet is incorporated into lactose broth than when lactose broth is used without an inhibiting agent. This work was done by Hall and Ellefson,^{8, 9} who reported that the percentage of samples positive for *B. coli* was not lowered.

Petroff's medium for culture of the tubercle bacillus employs gentian violet in a concentration of 1:10,000 to discourage growth of molds and other common contaminants. The fact that acid-fast organisms, although gram-positive, are highly resistant to gentian violet, was first noted by Churchman¹⁰ and has been elucidated by the work of Stearn and Stearn¹¹ who found that tubercle bacilli decompose gentian violet.

The dye, incorporated in blood agar plates, was used in a wound study reported by Francis,¹² who found in 300 swabs from burns and wounds inoculated in parallel on plain blood agar and gentian violet-blood agar that 32.7 per cent were positive for beta hemolytic streptococci when the dye was present, whereas, only 22.7 per cent were positive otherwise.

Gentian violet is known to be of definite aid in purifying anaerobes when their cultures are contaminated with *B. subtilis*, and has been used by Hall¹³ routinely as an aid in isolation of anaerobes.¹⁴ No reports, however, have been made of the numbers and types of anaerobes isolated in a series of cultures where gentian violet medium was compared with a dye-free medium.

From the Harrison Department of Surgical Research, Schools of Medicine, University of Pennsylvania, Philadelphia; and the City Hospital of Akron, Akron, Ohio.

The work described in this report was done under a contract recommended by the Committee on Medical Research between the Office of Scientific Research and Development and the University of Pennsylvania, and is part of the Contaminated Wound Project of the Subcommittee on Surgical Infections.

Received for publication, June 16, 1944.

REFERENCES

1. Churchman, J. W.: Purification of Cultures of Bacteria by Means of Reverse Selective Bacteriostatic Properties of Aniline Dye, *Proc. Soc. Exper. Biol. & Med.* 23: 530, 1926.
2. Farley, D. L.: The Cultivation of *Epidermophyton Inguinale*, *Arch. Dermat. & Syph.* 2: 466, 1920.
3. Farley, D. L.: The Use of Gentian Violet as a Restrainer in Isolation of Pathogenic Molds, *Arch. Dermat. & Syph.* 2: 459, 1920.
4. Bernstein, E. P., and Lowe, L.: A Simple Method for Isolation of the Influenza Bacillus, *J. Infect. Dis.* 24: 79, 1919.
5. Thomas, J. C.: Reduction of Dust-borne Bacteria by Oiling Floors, *Lancet* 2: 123, 1941.
6. Thomas, J. C., and Van den Ende, N.: The Reduction of Dust-borne Bacteria in the Air of Hospital Wards by Liquid-paraffin Treatment of Bedclothes, *Brit. M. J.* 1: 935, 1941.
7. Bourdillon, R. B., Lidwell, O. M., and Thomas, J. C.: A Slit-sampler for Collecting and Counting Air-borne Bacteria, *J. Hyg. (Camb.)* 41: 197, 1941.
8. Hall, I. C., and Ellefson, L. J.: The Elimination of Spurious Presumptive Tests for *B. coli* in Water by Use of Gentian Violet, *J. Bact.* 3: 329, 1918.
9. Hall, I. C., and Ellefson, L. J.: Further Studies on Gentian Violet as a Means of Eliminating Spurious Presumptive Tests for *B. coli* in Water, *J. Am. Water Works A.* 6: 67, 1919.
10. Churchman, J. W.: The Selective Bactericidal Action of Gentian Violet, *J. Exper. Med.* 16: 221, 1912.
11. Stearn, Esther, and Stearn, Allen: Anomalous Behavior of Tubercle Bacilli as Gram-Positive Organisms With High Resistance to Gentian Violet, *J. Bact.* 23: 399, 1932.
12. Francis, A. E.: Isolation of Hemolytic Streptococci From Wounds, *Lancet* 2: 159, 1941.
13. Hall, I. C.: Selective Elimination of the Hvy Bacillus From Cultures of Obligate Anaerobes, *J. A. M. A.* 72: 274, 1919.
14. Hall, I. C.: Practical Methods in the Purification of Obligate Anaerobes, *J. Infect. Dis.* 27: 576, 1920.
15. Garrod, L. P.: The Selective Bacteriostatic Action of Gentian Violet, *Brit. M. J.* 1: 290, 1942.
16. Fleming, A.: A Simple Method for Using Penicillin, Tellurite and Gentian Violet for Differential Culture, *Brit. M. J.* 1: 574, 1942.

clostridia was done at the central laboratory of the Office of Scientific Research and Development wound-study project under the direction of Ivan C. Hall.

Table I shows that the total number of species isolated is greatly augmented by the duplicate culture on a gentian violet plate. On the whole, the results are in accord with the early studies of Churchman¹⁰ on species susceptibility to the dye and the more recent work of Garrod¹⁵ on the same subject. *B. subtilis* was effectively inhibited; in the instances where it did occur on the gentian violet plates, its growth was sufficiently retarded to aid in isolating neighboring colonies.

In a study of the type here conducted, gentian violet agar could not be used as the only medium, since we were interested in isolation of the staphylococci, which, next to *B. subtilis*, was the organism most consistently inhibited. In fact, appearance of organisms on gentian violet agar, when they were absent on plain blood agar, can largely be attributed to inhibition of the vigorously growing *B. subtilis* and the anaerobic sporeformers, and inhibition of staphylococci and diphtheroids, all organisms which may prevent growth of streptococci and other slowly growing bacteria. In the case of anaerobic cultures, gentian violet served the additional useful role of preventing the spreading of the motile clostridia, which also tend to monopolize the surface of an agar plate.

Gentian violet proved of more aid in isolation of *Cl. welchii* than the figures here would indicate. Early in the study it was found to be so useful that whenever stormy fermentation occurred in iron-milk, also used routinely for every specimen, the milk was plated only on a gentian violet-blood agar plate.

Since *Cl. tetani* was not isolated in this series, we cannot here answer the question of whether gentian violet would render more difficult the primary isolation of this organism. On the whole no indication was found that any one species of clostridium could not be readily isolated on gentian violet medium.

The large number of anaerobic staphylococci isolated are of interest for several reasons. They obviously were not affected by the concentration of dye used and their relatively high total incidence may be attributed to the fact that they grow out only after four to seven days, a time when the plain plates were often overgrown. Bergey's *Manual of Determinative Bacteriology*, 1939, lists only three species of anaerobic staphylococci and the majority of strains here isolated did not fit the description of any of the three.

Although complete plates were used as the duplicates in this study, similar results might have been obtained with the use of gentian violet spread over half the surface of a blood agar plate, as recommended by Fleming.¹⁶

SUMMARY

Blood agar plates containing gentian violet used in duplicate with blood agar plates without dye, permitted the isolation of many aerobes and anaerobes which would have been missed had the inhibiting medium not been employed. Such results appear to be due to the inhibition by gentian violet of the rapidly growing staphylococci and aerobic sporeformers, and to the fact that clostridia give less spreading overgrowth with this medium.

POLYCHROME NO. 28

A SIMPLIFIED POLYCHROME STAIN FOR USE IN THE CLINICAL LABORATORY

CHARLES F. ELVERS

IN 1943 a preliminary report was published on a single process polychrome stain¹ designed primarily for use in staining spermatozoa and material encountered in specimens of prostate secretion. Later the stain was used routinely in the laboratories of the James Buchanan Brady Urological Institute of the Johns Hopkins Hospital to determine the presence or absence of bacteria, cells, mucus, lecithin, etc., and to differentiate between other normal and pathological materials. A considerable amount of this stain was also supplied to other institutions and laboratories. The numerous requests for reprints of this article received during the past year indicate that there is a definite need for a single process differential stain of this type, and experimental work was continued by modification of the original formula with the idea of simplifying the stain and adapting it for general use in the clinical laboratory.

In attempting to prepare a stain of this type, one must remember that while urine specimens are normally acid, prostatic secretion may show a hydrogen ion concentration varying from pH 6.5 to pH 8.0 or higher, and that dyes generally employed to demonstrate the presence or absence of leucocytes, epithelium, mucus, and most bacteria, fail to effectively stain fats, lecithin, spermatozoa and other seminal vesical elements. The miscibility of any compound stain with such a variety of material is of first importance.

The rate of acid formation in cytological material, as noted by Geschickter,² is more rapid than is generally supposed, and without some corrective measure uniformity of staining is not possible. In the past the technique in general use required a treatment by a pre-staining bath, either a mordant or buffer solution to reduce the thymonucleic and para-amino acids present as a result of cell degeneration. While this is effective as a control of the pH, its use imposes an additional and time consuming operation in the routine examination of clinical material, and except in research work is seldom carried out.

The simple or basic stain consisting of an aqueous-alcoholic solution of methylene blue has long been employed as a short cut in routine work. Simple basic stains of this type stain the cellular nuclei and most bacteria, but fail to differentiate between other cellular elements, and, therefore, have little value except to determine the number of bacteria present and their relation to the amounts of pus or exudate.

Polychrome No. 28, like the previous stain (No. 27) possesses the fundamental requirements of being a single process stain. It is rapid in action, produces minimal shrinkage of cells, requires no pre-staining baths, mordant,

From the Laboratories of John Wyeth and Brother Division, Wyeth Incorporated, Philadelphia, Pa.

Received for publication, April 14, 1944.



Fig. 1.—A, Centrifugalized urine specimen. B, Prostatic and seminal vesicle secretion. Stained by Polychrome No. 28, Elvers.

buffer, or decolorization and counter stain. The technique for its use is simple and completed in one action.

Delicate reactions such as those produced in the polybroming of Romanowsky and similar stains have been avoided, and in Polychrome No. 28 the differential staining reaction is effected by the use of a balanced formula of basic dyes in a buffered solvent. The neutrophilic, oxyphilic and basophilic elements react with that portion of the dye for which each has the greatest affinity, and this selective and contrasting color combination readily permits the identification of histological structures. Thin, even smears are essential for good staining and clear definition. Fresh material gives better results than that which has been allowed to dry for several hours, but slides which have been kept in a closed jar containing equal parts of 95 per cent ethyl alcohol, and of ether will stain well even after several weeks.

Freshly expressed prostatic secretion tends to become viscous on exposure to air. This viscosity precludes preparation of uniformly thin films, but if kept at oven temperature (56° to 58° C.) for ten to fifteen minutes the mucin will break down and the material may then be evenly spread on the micro slide.

The buffered solvent (stock) is made as follows:

Disodium phosphate	29 Gm.
Citric acid, C.P.	9.5 Gm.
Glycerin	40 ml.
Ethyl alcohol, 95 per cent	150 ml.
Distilled water q.s. to 1 liter.	

The pH of the above will be approximately 5.0, but may be measured potentiometrically and, if required, may be adjusted by adding small amounts of acid or disodium phosphate.

The stain is made by adding

- 2 Gm. of tetra-methyl thionin and
- 0.2 Gm. of rosaniline hydrochloride to

98 ml. of the pH 5.0 buffered solvent; stirring until all dye is dissolved; allowing to stand for twenty-four hours, and filtering or straining through cotton. Stain samples made by this formula still give sharp color contrast and good definition after eighteen months.

TECHNIQUE FOR USE

1. Fix thin, even smear to slide by heat or other method as desired, and cool to room temperature.
2. Cover smear with stain and allow to remain 30 to 60 seconds; slightly longer if deeper color is desired.
3. Rinse gently in distilled water, air dry and examine.

Mucin, cell cytoplasm, etc., are stained pink, nuclear material is blue. Cells which have lost their biochemical reaction due to degenerative changes are indefinitely stained a faint purplish red, the nucleus being darker than the cytoplasm. The front half of the head of spermatozoon remains clear or is

faintly stained, the back portion being dark blue, the spiral axillary thread and tail portion are red. Due to the fact that the tail structure is stained throughout its entire length, it appears much longer than when stained by other methods. Lecithin and cornified epithelial cells are bright red, bacteria are deep blue to purple.

Contrasting colors of the different elements are bright and well defined.

REFERENCES

1. Elvers, C. F.: Elvers Polychrome, J. Urol. 49: 747, 1943.
2. Geschickter, C. F.: Stain Technol. 6: 5, 1931.

SELF-BUFFERING STAINING SOLUTIONS*

WITH SPECIAL REFERENCE TO WRIGHT'S POLYCHROME METHYLENE
BLUE-EOSIN STAIN

BERNARD WITLIN, Sc.D.,† HONOLULU, T. H.

INTRODUCTION

POLYCHROME methylene blue-eosin stains have been widely used throughout the United States. Varying results, however, have been obtained in different laboratories employing the same staining solution.¹

Kalthoff,² French,³ Haynes,⁴ Smith,⁵ Coun and Margolena,⁶ and Kingsley⁷ suggested practical methods for obtaining uniform results with stains by the use of buffer solutions. McDunkin⁸ demonstrated that much of the trouble encountered with polychrome stains could be eliminated if a phosphate buffer solution (pH 6.4) was used instead of distilled water in the staining procedure.

It was noted by the author that aqueous phosphate buffer solutions were subject to bacterial decomposition and that the addition of bacteriostatic substances in sufficient quantities to inhibit a biologic breakdown of the buffer solution, materially affected the efficiency of the Wright's staining solution. In an effort to offset the inconvenience of making buffer solutions at semimonthly intervals and effect an economy in reagents while maintaining staining efficiency, a study was made of the efficacy of buffering the methyl alcohol solution of Wright's stain.

EXPERIMENTAL

A. Methyl Alcohol Buffer.—Since Wright's stain is usually dissolved in methyl alcohol, it was decided to find methyl alcohol-soluble chemicals which were capable of buffering distilled water as commonly employed in the laboratory. Such a buffer system was found in sodium acetate and acetic acid.[‡] These chemicals were readily soluble in methyl alcohol and/or in distilled water. The principal premise was that there would be practically no ionization when Wright's stain was dissolved in such a methyl alcohol-sodium acetate-acetic acid[‡] solution. Ionization, however, would take place upon the dilution of the stain with distilled water and sufficient buffering quality would be released with the dissociation of the hydrogen ions in the aqueous solution.

Various concentrations and formulas based upon molar solutions of these chemicals were prepared which would give a pH of 6.4-6.6 (colorimetrically

*This research was begun at the Philadelphia College of Pharmacy and Science.

†Bacteriologist, Territory of Hawaii, Board of Health, Territory of Hawaii P. A. Sanitarian (R), U. S. Public Health Service, Bureau of States Service assigned to the Board of Health, Territory of Hawaii

‡Theoretically, acetic anhydride was considered in place of the acetic acid, but practically, a chemically pure grade of glacial acetic acid possesses insufficient water to materially effect ionization in the Wright's stain sodium acetate-acetic acid solution.

Received for publication, July 7, 1944.

and potentiometrically) when diluted with distilled water. The most suitable preparation of methyl alcohol buffer-Wright's stain solvent was as follows:

One hundred and twenty milligrams of $\text{NaC}_2\text{H}_3\text{O}_2 \cdot 3\text{H}_2\text{O}$ (sodium acetate—Merck's reagent) were dissolved in 100 c.c. of CH_3OH (methyl alcohol, free from acetone—Merck's reagent). To this was added 0.4 c.c. of a solution made by dissolving 0.1 c.c. of glacial acetic acid (Baker's C.P.) in 10 c.c. of methyl alcohol (Merck's reagent, free from acetone). The total amount of acetic acid in 100 c.c. of the sodium acetate-acetic acid-methyl alcohol solution is 0.004 c.c.

B. Staining Solution.—In every 60 c.c. of the methyl alcohol-sodium acetate-acetic acid solution, 0.1 Gm. of Wright's stain was dissolved. After 24 hours the staining solution was filtered. The buffering effect of this staining solution kept the pH approximately 6.4 to 6.6 when an equal volume of distilled water was added after the undiluted methyl alcohol-staining solution had first fixed the blood film.

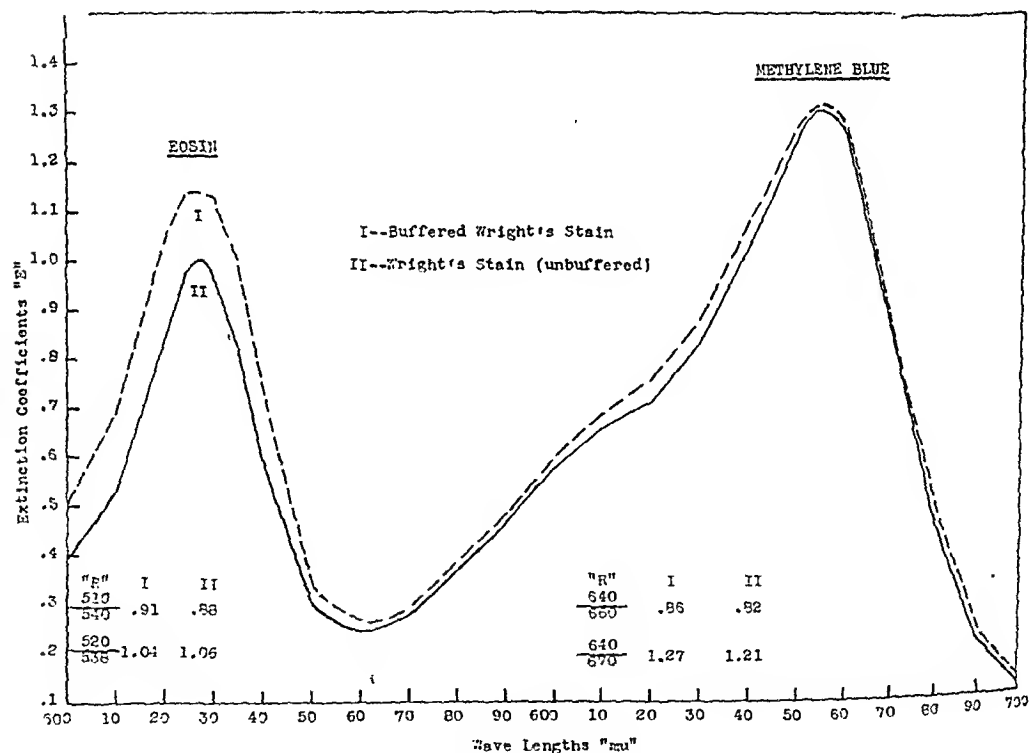


Fig. 1.—Extinction coefficient of Wright's stain.

C. Staining Technique.—Fifteen drops (about 0.35 c.c.) of Wright's stain were applied to the blood film and permitted to act for two minutes. Fifteen drops (about 0.75 c.c.) of distilled water were then added and three minutes were allowed for staining. The slide was rinsed in running water for 30 seconds, drained, and dried.

Comparison of Self-Buffered Wright's Stain With Nonbuffered Wright's Stain.—In November, 1938, sixteen different samples of commercially prepared Wright's stain were dissolved in duplicate sets; one series was made in buffered

methyl alcohol as discussed above (A and B), the other in methyl alcohol alone.

Numerous peripheral blood smears from normal individuals, as well as from patients suffering from malaria, filariasis, leprosy, leucemia, and pernicious anemia were prepared. Series of these blood smears were stained at monthly intervals for five years with the above staining solutions, employing distilled water as the diluent for the buffered stains and phosphate buffer solutions as the diluent for the unbuffered stains. In all instances the self-buffered Wright's stain solutions followed by the dilution with distilled water produced results comparable to the nonbuffered series. In not one instance has a buffered stain solution broken chemically or failed to stain properly. Final concentrations up to 300 mg. of sodium acetate and 0.01 c.c. of glacial acetic acid in 100 c.c. of methyl alcohol gave satisfactory results. An increase in the chemical concentrations, however, affected the erythrocytes from the cases of pernicious anemia. It was therefore deemed advisable to lower the chemical buffer concentration to a safe limit. This was 120 mg. of sodium acetate and 0.004 c.c. glacial acetic acid per 100 c.c. of methyl alcohol. In all instances the self-buffered stain solutions have resulted in good color detail. The differentiation of the cytoplasm of leucocytes and monocytes has been excellent as has been that of neutrophilic granules, eosinophilic granules, basophilic granules, blood platelets, and erythrocytes.

D. *Extinction Coefficient*.—Extinction coefficients were determined in the laboratory of the Biological Stain Commission in cooperation with the commission's technician, Mrs. A. P. Bradshaw in order to make certain that the stain itself was not altered by the buffering technique. The graphic results of the findings are presented in Fig. 1. The "extinction coefficients" of the eosin and methylene blue were not materially altered by the addition of the buffers to the dye solution. The changes in hue were so slight that no difference in staining due to this change would be expected.

SUMMARY AND CONCLUSION

A convenient and practical method for buffering methyl alcohol solutions of polychrome methylene blue-eosin stain is presented. The detailed method of preparation is described.

REFERENCES

1. Conn, H. J.: Personal communication, Oct. 17, 1940.
2. Kalthoff: Chem. Weekblad 24: 78, 1927.
3. French, R. W.: Practical Methods for the Control of Hydrogen-Ion Concentration in Staining Procedure—the Use of "Buffers," Stain Technol. 5: 87, 1930.
4. Haynes, Rachel: Investigation of Thiazin Dyes as Biological Stains. II. Influence of Buffered Solutions on Staining Properties, Stain Technol. 3: 131, 1928.
5. Smith, Ralph B.: pH Determination in Alcoholic Solutions, J. Am. Pharm. A. 17: 241, 1928.
6. Conn, H. J., and Margolena, Lubov A.: Notes on Technique—Difficulties Encountered in Obtaining a Satisfactory Wright Stain, Stain Technol. 8: 35, 1933.
7. Kingsley, D. M.: A New Hematological Stain, Stain Technol. 10: 127, 1935.
8. McJunkin, F. A.: A Benzidin-Polychrome Stain for Blood, J. A. M. A. 74: 17, 1920.

A MECHANICAL DEVICE FOR MAKING MULTIPLE PARAFFIN RINGS FOR SLIDE PRECIPITATION TESTS FOR SYPHILIS

JOHN W. NEUFELD, A.B., AND MALCOLM H. MERRILL, M.D., BERKELEY, CALIF.

IN A laboratory where a large number of slide precipitation tests for syphilis are run, the making of paraffin rings individually by hand is a tedious and time-consuming procedure. To eliminate these irksome factors the machine described here was devised. In the California State Public Health Laboratory, where this device was first put into operation, between four and five thousand Kline tests were done per day at the height of Selective Service activity. It required between five and six man-hours to make this many rings by hand, whereas with the machine that work can be completed in about 45 minutes.

The machine is based on the printing-press principle, with the desired number of paraffin rings being "printed" onto a glass plate in one operation. The size of the plates and the number of rings on each plate can be suited to individual requirements. We have found a plate 5 x 12 inches, accommodating 4 rows of 13 rings each, to be satisfactory. This provides for 50 tests together with positive and antigen controls. There is ample space for numbering.

The ring maker can be made for the most part of wood. The only metal required is as follows: about 4 feet of flat iron $\frac{3}{16}$ x $\frac{3}{4}$ inches, a metal rod $\frac{3}{4}$ inches in diameter by 16 inches long, and the metal paraffin pan.

The machine is shown in Fig. 1. The electric hot-plate *A* should be thermostatically controlled, but we have found an ordinary plate such as the General Electric Hotpoint (Cat. #40104) with three heat controls to be satisfactory. Maintaining the paraffin at the proper temperature is very important. This should be between 105°-110° C., at which temperature the paraffin smokes slightly. This temperature is held fairly well with the switch at "low."

The pan for the paraffin *B* can be made of 16 or 20 gauge galvanized sheet metal. The corner seams must be smooth to avoid leakage over the edge by capillary attraction. It is held in place by means of the spring elips *C*. The depth of the pan should be at least one inch and the length and width slightly greater than the corresponding dimensions of the printing head *M*.

The post *D* is made of $\frac{3}{4}$ inch round iron, and is about 16 inches long. It is fastened into the base *E* at the point of intersection of two lines perpendicular to the long axis of, and drawn from the mid-points of, the paraffin pan *B* and the platform *P*.

The swivel head *F* fits over the post *D* and is held in place by the platform *G* and the collar *H*. The stops *I* (there is one also on the opposite side, not visible) are driven into the platform *G* in the proper positions so as to allow the swivel motion through exactly 90°.

From the Division of Laboratories, California State Department of Public Health.
(Received for publication, June 27, 1944.)

The lever arms *J* and *K* are fastened onto the swivel head *F* as shown. The distances *X-Y* and *X'-Y'* must of course be identical and will be between 7 and 9 inches depending on the size and shape of the glass plates used. The upper lever arm *J*, with the handle, should be about twice as long as the lower arm *K*. The brackets *L* fasten to the lever arms and screw into the printing head *M* as shown. The spring *S* must be quite strong to lift the heavy printing head.

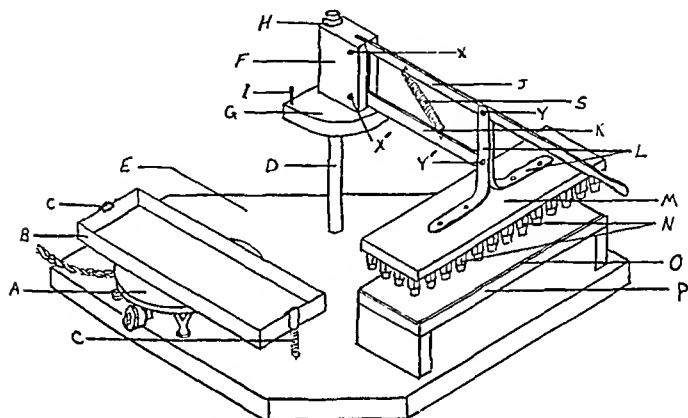


Fig. 1.—For explanation see text.

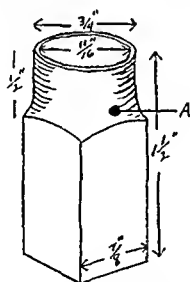


Fig. 2.—For explanation see text.

The printing head *M* is $\frac{3}{4}$ inch hard wood or 5-ply wood. It must not have any warp. The other dimensions depend on the size of glass plate used. The individual ring blocks *N* are fastened to it; these are described in detail below.

The platform *M* on which the glass plate *O* rests should be the same size as the platform *P*. This makes it easy to place the plate in exactly the same

position each time. The height of the platform must approximate that of the top edge of the paraffin pan.

The printing blocks *N* (enlarged in Fig. 2) are made of hardwood (oak or maple is suitable) and must be turned out on a precision lathe. The blocks are $1\frac{1}{2}$ inches long by $\frac{7}{8}$ inches square. An $1\frac{1}{16}$ -inch hole is first drilled into the exact center of the block to a depth of about $\frac{3}{4}$ inch. Then the outside is milled down to a diameter of $\frac{3}{4}$ inches leaving a tubular end with $\frac{1}{32}$ -inch walls. This tubular part should not be more than $\frac{1}{2}$ to $\frac{5}{8}$ inches long to avoid warping. The use of square-shaped blocks also helps to prevent warping. Screw holes are drilled through the center of the square end of the blocks and fastened to the printing head *M* with a snug but not tight fitting screw. A $\frac{1}{8}$ -inch air hole *A* must be drilled through the wall as shown in Fig. 2.

The greatest difficulty was encountered in getting all blocks to print evenly and in eliminating "misses." This was "cured" by screwing all blocks tight, sanding the tubular edges on a large, even-surfaced mechanical sander, and then loosening the screws about half a turn to allow a little play for self-adjustment. The sandpaper used must be very fine to avoid tearing the thin walls. All burrs must be sanded off the tube edges as the final step.

SUMMARY

A mechanical device is described for making multiple paraffin rings used primarily in serological tests for syphilis such as those devised by Kline and Mazzini.

A similar device has been used in the California State Department of Health Laboratory for over a year and has been found to save considerable time and to produce more uniform rings than are ordinarily made by hand.

It is reasonably inexpensive, as it can be made largely of wood, but its construction requires some precision machinery and considerable mechanical skill.

A SIMPLE ACID WATER SOLUTION FOR BETTER VISUALIZATION OF MALARIAL PARASITES USING WRIGHT'S STAIN FOR THICK BLOOD FILMS

MARGUERITE L. GRAU, BILOXI, MISS.

CONSIDERABLE difficulty has been encountered in obtaining satisfactory differentiation of malarial parasites in thick blood films stained with Wright's stain. Either distilled water or buffered solutions containing monobasic potassium phosphate and dibasic sodium phosphate was used to dilute the stain and to wash it from the slide. The same technique, when applied to ordinary blood films, gave excellent cellular detail. Giemsa stain gives good differentiation of plasmodia in thick films, but since it is less commonly available than is Wright's stain, any alterations in technique which would permit the use of Wright's stain would have definite practical value. This note reports that good results may be obtained on thick blood films if: (1) the red blood cells are hemolyzed in a fixing solution rather than in water, and (2) an acid water is used to dilute the Wright's stain and to wash the slide.

The variations in techniques were made on thick blood films known to contain malarial parasites. Dilute acids, alkalis, and buffered solutions with a pH range of from two to ten were tried as diluents for the Wright's stain. An acid water, prepared by adding 0.06 c.c. (2 drops) 0.1 N HCl to 100 c.c. of distilled water gave excellent results. Clearer detail and less distortion of plasmodia were obtained if the blood was hemolyzed in a fixing solution rather than in water. The fixing solution was made by adding 1 c.c. of glacial acetic acid to 99 c.c. of 5 per cent formalin immediately prior to use. If the solution was kept in a covered staining jar, it was satisfactory for a large number of slides.

The dried thick blood film was immersed in the fixing solution for 10 minutes and then washed in two changes of distilled water for 5 minutes each time. After the slide had thoroughly dried in air, the film was covered with Wright's stain for 1 minute. An equal amount of acid water was then added and 10 minutes were allowed for staining. The slide was washed with acid water, dried, and examined.

With preparations treated in this manner, ring-form trophozoites show clearly against a dusky blue background. The cytoplasmic circle is a sky blue, while the chromatin dot is definitely red and easily detected under the microscope. Developing forms, schizonts, and gametocytes are also distinct.

From the Bacteriology Laboratory, U. S. Veterans Administration, Biloxi, Miss.
Published with the permission of the Medical Director, Veterans Administration, who assumes no responsibility for the opinions expressed or the conclusions drawn by the author.
Received for publication June 19, 1944.

CHEMICAL

A MICRO-MINCE TO FACILITATE PREPARATION OF TISSUE EXTRACTS

W. C. ALFORD, B.S., AND E. D. PALMES, M.S., BETHESDA, MD.

THE accurate determination of various biologically important substances in animal tissue depends largely on the degree of comminution of the sample, since intimate contact between the tissue and the extracting liquid is essential. The mortar and pestle are used to grind small tissue samples, but this method is laborious and quantitative transfers are difficult. Mechanical apparatuses have been proposed for this purpose by Corper and Cohn¹ and Potter and Elvehjem,² but these are somewhat different in principle from the apparatus herein proposed. The Waring Blendor, which is more similar in principle, is not adaptable to tissue samples of the size under consideration. The proposed micro-mincer is shown in Fig. 1.

Description.—The motive power for the mincer is supplied by a Precision "Aero-Mix" air-driven motor. Under actual operating conditions the speed is from 5,500 to 6,000 r.p.m. The shaft and cutting blades are made of chromium-plated steel. As shown in Fig. 1, the blades have no pitch, but two opposing blades are bent at an angle of about 30 degrees from the plane normal to the shaft. The end of the shaft is drilled and tapped and the blade is held in place by means of a chromium-plated bolt. The four leading edges of the blades are sharpened.

An ordinary round bottom 50 ml. centrifuge tube is used to hold the sample and extracting liquid. During the mincing operation the tube is held in a wooden block which fits snugly in the stand.

Operation.—From $\frac{1}{10}$ to 6 Gm. of tissue and 5 to 10 ml. of the extracting liquid are introduced into the 50 ml. tube. The tube is placed in the wooden holder and the holder raised to the stop. In this position the blade is approximately 0.5 inches from the bottom of the tube, and the holes in the stand and holder coincide. The metal pin is inserted to anchor the holder.

To avoid spattering, the mincing is begun with the motor running at slow speed. The speed is then increased to maximum and the mincing continued for from fifteen seconds to one minute. The motor is stopped, the pin removed, and the holder lowered so that the blade is clear of the mixture. The motor is now turned on full speed momentarily in order to throw any mixture adhering to the shaft and blade onto the side wall of the tube. The holder is lowered and the tube removed. When fibers are caught on the blade, it is very simple

From the Industrial Hygiene Research Laboratory, National Institute of Health, U. S. Public Health Service.

Received for publication, Aug. 15, 1944.

to remove them with a pair of forceps and place them in the tube with the minced tissue.

The shaft and blade are washed by placing from 5 to 10 ml. of the extracting liquid in another centrifuge tube and using the same procedure as that used for mincing the tissue, except that the motor is brought to full speed immediately and run for only a few seconds. The wash liquid is combined with the original mixture.

The extract may now be separated from the tissue by centrifuging in the same tube.

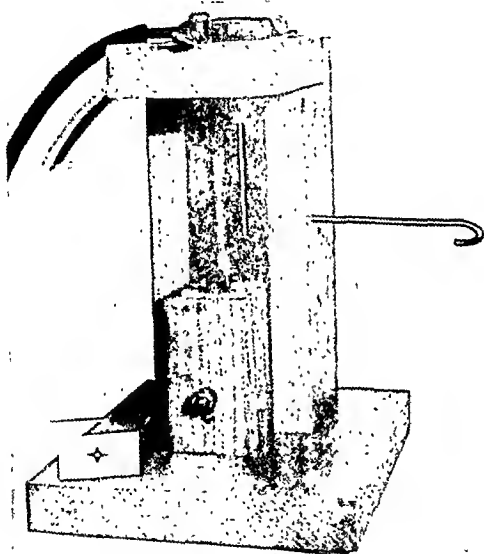


Fig. 1.—Micro-mincer with extra blade.

Use.—In the course of an investigation of the vitamin C content of guinea pig organs undertaken in this laboratory, considerable time and labor were saved by using the apparatus described herein for preparing trichloroacetic acid extracts. The kinds of tissues and approximate amounts of each used for these determinations were as follows: liver, 3.5 to 6.0 Gm.; kidney, 3.0 to 4.5 Gm.; spleen, 0.5 to 2.0 Gm.; adrenals, 0.1 to 0.3 Gm. Because of the small size of the adrenals, it was necessary to add a small amount of an inert material to the tissue so that the bulk of solids would be sufficient to insure satisfactory mincing. Paraffin shavings were found to be suitable for this purpose. Results obtained using this apparatus were in excellent agreement with those obtained when a mortar and pestle were used for grinding the tissues.

SUMMARY

A new micro-mincer is proposed to facilitate the preparation of extracts of small animal tissues. It is simple to construct and operates efficiently.

We wish to express our appreciation to Passed Assistant Surgeon Glen E. Ogden for determining the speed of the mincer.

REFERENCES

1. Corper, H. J., and Cohn, M. L.: A Mechanical Device for Preparing Fine Suspensions of Tubercle Bacilli and Other Micro-Organisms, *J. LAB. & CLIN. MED.* 21: 428, 1936.
2. Potter, V. R., and Elvehjem, C. A.: A Modified Method for the Study of Tissue Oxidations, *J. Biol. Chem.* 114: 495, 1936.

BOOK REVIEWS AND NOTICES

The Art of Anesthesia. By *Paluel J. Flagg*, M.D., Visiting Anesthetist to Manhattan Eye and Ear Hospital; Consulting Anesthetist to St. Vincent's Hospital, New York, N. Y.; Consulting Anesthetist to Woman's Hospital, Sea View Hospital, Jamaica Hospital, Mount Vernon Hospital, Flushing Hospital, Mary Immaculate Hospital, St. Mary's Hospital, Far Rockaway, N. Y., Nassau Hospital, Long Island; Director of Pneumatology, World's Fair, New York, N. Y. and Chairman on Asphyxia of the American Medical Association. Seventh edition, J. B. Lippincott Co., Philadelphia. Price \$6.00. Cloth with 519 pages and 166 illustrations.

Manual of Urology. By *R. M. LeComte*, M.D., F.A.C.S., Professor of Urology, Georgetown University Medical Department, Member of the American Urological Association. Third edition, The Williams & Wilkins Co., Baltimore. Price \$4.00. Cloth with 305 pages.

Fundamentals of Internal Medicine. By *Wallace Mason Yater*, A.B., M.D., M.S. in (Med.), F.A.C.P., Professor of Medicine and Director of the Department of Medicine, Georgetown University Hospital; Physician-in-Chief, Gallinger Municipal Hospital, Washington, D. C.; Formerly, Fellow in Medicine, the Mayo Foundation. Second edition, D. Appleton-Century Co., New York. Price \$10.00. Cloth with 1,204 pages.

Fertility in Men. By *Robert Sherman Hotchkiss*, B.S., M.D., Lieutenant Commander, M.C., U.S.N.R. (on active service); Assistant Professor of Urology, New York University Medical College; Instructor in Surgery (Urology), Cornell Medical College; Assistant Visiting Attending Physician, Department of Urology, Bellevue Hospital; Assistant Visiting Attending Physician in Surgery (Urology), New York Hospital; Chief of Urological Clinic, New York University Medical College Clinic. Cloth with 216 pages and 95 illustrations.

Fertility in Women. By *Samuel L. Siegler*, M.D., F.A.C.S., Attending Obstetrician and Gynecologist, Brooklyn Women's Hospital; Attending Gynecologist, Unity Hospital, Assistant Obstetrician and Gynecologist, Greenpoint Hospital; Attending Sterility Clinic, Greenpoint Hospital; Consultant in Gynecology, Rockaway Beach Hospital; Diplomate of American Board of Obstetrics and Gynecology; Fellow of New York Academy of Medicine; Member of Society for the Study of Internal Secretions. Cloth with 450 pages and 194 illustrations. J. B. Lippincott Company, Philadelphia. In slip case, \$8.00.

Quick Reference Book for Medicine and Surgery. By *George E. Rehberger*, A.B., M.D. Twelfth edition, J. B. Lippincott Co., Philadelphia. Price \$15.00. Cloth with 1,460 pages.

Elements of Medical Mycology. By *Jacob Hyams Swartz*, M.D., Assistant Professor of Dermatology, Harvard Medical School (Post Graduate School); Member of American Dermatological Association and American Mycological Association; Dermatologist, Massachusetts General Hospital. Grune and Stratton, Inc., New York. Price \$4.50. Cloth with 179 pages.

Allergy. By *Erich Urbach*, M.D., Chief of Allergy Service, Jewish Hospital of Philadelphia; Assistant in Dermatology, University of Pennsylvania School of Medicine; Member of Board of Regents, American College of Allergists. Grune and Stratton, Inc., New York. Cloth with 1,073 pages.

Practice of Medicine. By *Jonathan Campbell Meakins*, M.D., LL.D., Brigadier Deputy Director General of Medical Services, Royal Canadian Army Medical Corps; Professor of Medicine and Director of Department of Medicine, McGill University; Physician-in-Chief, Royal Victoria Hospital, Montreal; Formerly Professor of Therapeutics and Clinical Medicine, University of Edinburgh; Fellow of Royal Society of Physicians of London; Fellow of Royal College of Physicians, Edinburgh; Honorary Fellow of Royal College of Surgeons of Edinburgh; Fellow of Royal College of Physicians, Canada; Fellow of American College of Physicians; Honorary Fellow of Royal Society of Medicine. Fourth edition, The C. V. Mosby Co., St. Louis. Price \$10.00. Cloth with 1,444 pages.

The Journal of Laboratory and Clinical Medicine

VOL. 29

NOVEMBER, 1944

No. 11

CLINICAL AND EXPERIMENTAL

CHOLINE DEFICIENCY STUDIES IN DOGS

J. M. MCKIBBIN, PH.D., S. THAYER, B.A., AND F. J. STARE, PH.D., M.D.
BOSTON, MASS.

THE importance of choline in the nutrition of the dog was suggested by the discovery of its lipotropic action on the liver of the depancreatized dog by Best, Ferguson, and Hershey in 1933.¹ Study of its deficiency in normal dogs has awaited the use of purified rations. Thus, Schaefer, McKibbin, and Elvehjem² demonstrated a lag in the early growth curve of young puppies fed a purified ration without choline. The addition of choline to this ration resulted in resumption of growth. Fouts³ fed young puppies and adult dogs on a diet high in fat and low in protein without added choline. With such a diet, he reported a fatty degeneration and cirrhosis of the liver. Hough, Monahan, Li, and Freeman⁴ observed an impaired hepatic dye clearance and high serum phosphatase values in dogs fed a diet low in protein and a diet low in methionine. This paper presents in detail some observations in the study of choline deficiency in young puppies.

EXPERIMENTAL

Litters of weanling mongrel puppies were used throughout the experiments. Upon receipt of the puppies at the laboratory, they were dewormed with Buchlorin and magnesium sulfate and placed on a diet of milk and Purina Dog Chow for a week or ten days' observation before the experiments were started. All dogs received subcutaneously 1.5 c.c. per pound of Pitman-Moore antidistemper serum each week.

Preliminary studies have shown inconsistencies in the growth of young puppies placed on the purified ration devised by Schaefer and co-workers.² One litter of animals grew well except for a slight lag in the early part of the

From the Department of Nutrition, Harvard School of Public Health, and the Department of Biological Chemistry, Harvard Medical School.

Received for publication, Sept. 8, 1944.

and also served as a control. It can be seen that this ration did not support growth in either the deficient or the control animals, although control Dog 2 made good progress for the first three weeks. At the end of the first week, oral doses of casein were given to Dogs 3 and 5 and dl-methionine to Dog 1 in an attempt to induce improvement in growth. However, there seemed to be no benefits from these additions. Evidently factors other than protein in nature were still not adequately met by this ration. In further attempts to improve it the following supplements were given to the choline-fed control dogs for varying periods: additional choline chloride; a mixture of the vitamins of the B-complex containing thiamine, pyridoxine, riboflavin, calcium pantothenate, and nicotinic acid; brewers' yeast; and a liver extract, Abbott's Liver Fraction "X." This liver extract fraction was the only supplement that produced any significant improvement. Response to this supplement was rapid and sustained for the duration of the experiment, as is shown in the growth curves of Dogs 2 and 6 in Fig. 1.

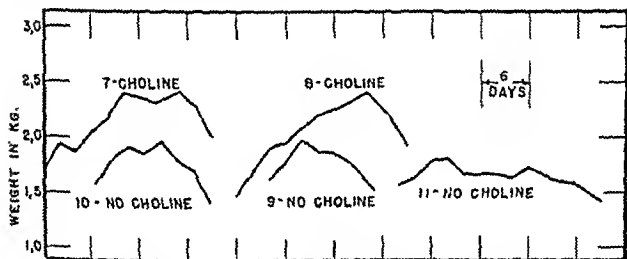


Fig. 2.—Growth curves of puppies of Experiment 2, Ration 2

With this experience another litter of five puppies (Experiment 2) was placed on Ration 2 (Table I). The growth curves of these puppies are shown in Fig. 2. It is apparent from the growth curves of Dogs 7 and 8, the control dogs receiving choline, that this ration would still not permit survival. The terminal findings for the animals in Experiments 1 and 2 will not be listed in detail since the deficiencies were complicated and have limited meaning in relation to deficiency of choline. It is of interest, however, that all the choline-deficient animals had extremely fatty liver, whereas the liver of the control dogs fed choline appeared normal. The average chloroform-extractable material found in the liver of Dogs 3, 4, 5, 9, 10, and 11 (receiving no choline) was 41.2 per cent. In the liver of Dogs 7 and 8, which had received choline, there was an average of 17.6 per cent chloroform-extractable material.

Experiment 2 indicated that at a concentration of 2 per cent the liver fraction used did not furnish enough of the missing factor or factors to render the control diet adequate. It was decided to try another liver extract low in choline and to use it in slightly higher concentration. The Wilson's Liver Fraction "L" is low in choline and is fairly potent in certain nutritional factors essential for the chick. This liver fraction therefore replaced the original liver fraction used in the ration and was included at a 3 per cent level (Ration

3, Table I). Three litters, totaling twelve puppies, were placed on this ration. The control puppies received in addition 150 mg. per cent of choline chloride. The growth performance of the first two litters (Experiment 3), totaling five puppies, is shown in Fig. 3. It can be seen from the curves that Dog 16 grew well throughout the experimental period of fifty-seven days. The littermate, Dog 15, receiving no choline did not grow at all for the first twelve days.

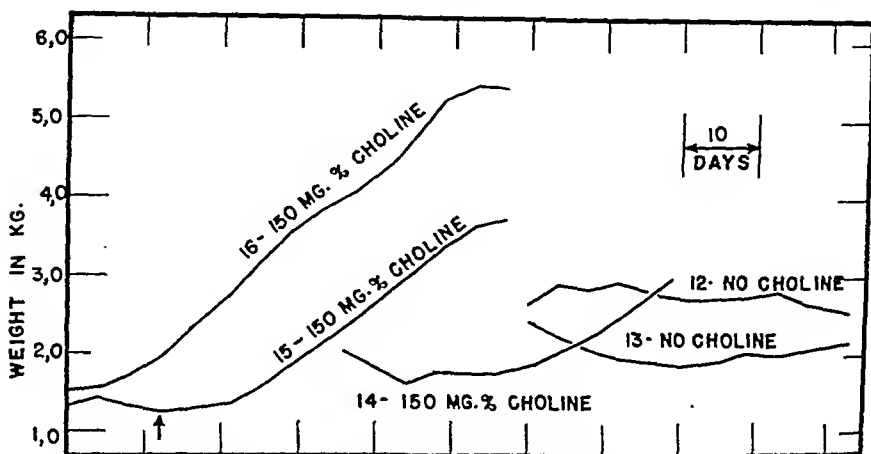


Fig. 3.—Growth curves of puppies of Experiment 3, Ration 3. Arrow indicates inception of supplement of 150 mg. choline chloride per 100 Gm. of ration.

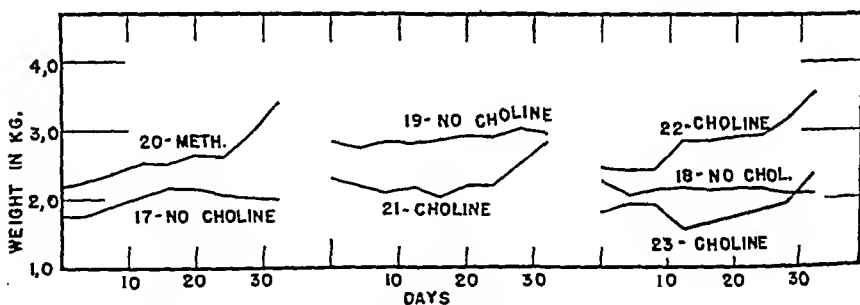


Fig. 4.—Growth curves of puppies of Experiment 4, Ration 3.

He was then given 0.5 Gm. choline chloride orally and then 150 mg. per cent were added to the ration. After a lag of ten days he began to grow and grew well thereafter. Dogs 12 and 13 received no choline and showed a net loss of weight over the experimental period of forty-three days. At this time they were sacrificed for tissue examination. Dog 14, a littermate of Dogs 12 and 13, received 150 mg. per cent of choline chloride. This dog failed to make any gain in weight until the twenty-eighth day. From this time on its weight and activity steadily improved until the forty-third day, when it was sacrificed for tissue examination.

The third litter placed on Ration 3 contained seven puppies (Experiment 4 and Fig. 4). Dogs 17, 18, and 19 were given the ration without additional supplements. The ration of Dog 20 was supplemented with 0.7 per cent dl-methionine, and that of Dogs 21, 22, and 23, with 150 mg. per cent choline

TABLE II

TERMINAL BLOOD AND TISSUE FINDINGS IN PUPPIES OF EXPERIMENTS 3 AND 4

DOG	SEX	RATION	SUPPLEMENT	DAYS ON EXPERIMENT	LIVER % CHCL ₃ EXTRACT	BROM-SULFALEIN TEST μ G. DYE PER ML. PLASMA	PLASMA PHOSPHATASE μ G. P LIBERATED		PROTHROMBIN TIME SEC.	HEMOGLOBIN GM. %	HEMATOCRIT %	RED BLOOD CELLS MILLION PER MM. ³
							8 HR.	24 HR.				
12	F	3	None	43	50.5	12	397	998	23.0	11.1	41.5	5.3
13	F	3	None	43	48.1	25	624	1,230	13.0	9.2	29.5	4.4
14	F	3	150 mg. % choline chloride	43	7.4	4	122	260	8.0	9.8	30.0	5.3
15	M	3	150 mg. % choline chloride	57	13.3	3	79	231	10.8	10.1	31.0	4.8
16	F	3	150 mg. % choline chloride	57	16.0	5	87	222	10.8	10.8	35.0	4.3
17	F	3	None	32	47.1	31	563	1,090	62.0	9.0	36.0	4.1
18	M	3	None	32	56.6	42	566	1,183	46.0	9.6	41.0	4.6
19	M	3	None	32	48.2	22	614	1,124	26.0	10.7	45.5	4.8
20	F	3	0.7% dl-methionine	32	11.9	10	82	245	13.7	10.6	39.0	5.8
21	M	3	150 mg. % choline chloride	32	19.1	4	137	349	10.5	10.1	37.0	5.1
22	F									11.0	40.0	7.0
23	M		100 mg. % choline chloride	32	12.5	5	140	300	6.0	9.3	30.0	4.8

chloride. Food consumption and growth in the animals receiving the supplemented rations were little better than in those on the basal ration for the first three weeks but showed improvement from this time until the thirty-second day, when all the puppies were sacrificed. Terminal hepatic dye clearance (bromsulfalein), plasma phosphatase, and prothrombin times were determined on all of the twelve dogs. These and other terminal findings are summarized in Table II.

In order to obtain information on the quantitative requirement of the growing pup for choline and on the biochemical nature of the deficiency, two more litters of puppies were placed on experiment. The first litter of seven puppies (Experiment 5, Fig. 5) was placed on Ration 4. In this ration, the liver extract was the same as that used in Ration 3, but the concentration was reduced to 2 per cent. Two of the dogs received no choline supplements, one received 5 mg. per 100 Gm. of ration, another 10 mg., two others 150 mg., and the last 0.7 per cent dl-methionine on the ration. Since the dogs did not eat the ration well, it was thought that more Liver Fraction "L" might be required, and they were all given a total of 11.5 Gm. per kilogram of body weight orally in the period from the seventeenth to twenty-first days. Striking improvement in the liver function tests of the two dogs with choline deficiencies occurred after this liver fraction feeding, and one of them (Dog 26) sacrificed at this time showed a relatively normal liver lipid content. Whether this liver repair was due to the 10 mg. of choline per kilogram of body weight obtained

TABLE
TERMINAL BLOOD AND TISSUE FINDINGS

DOG	SEX	RATION	SUPPLEMENT	DAYS ON EXPERI- MENT	LIVER % CHOL, EXTRACT	LIVER CHOL- ESTEROL MG. PER GM. DRY	BROM- SULFA- LEIN TEST µG. DYE PER ML. PLASMA	PLASMA PHOS- PHATASE µG. P LIBERATED	
								8 HR.	24 HR.
24	M	4	150 mg. % cho- line chloride	41	20.5		7	105	331
25	F	4	150 mg. % cho- line chloride	43	13.1		6	85	239
26	F	4	None	21	19.5		12	86	236
27	M	4	None	41	27.5		24	534	1,220
28	F	4	10 mg. % cho- line chloride	41	27.9		24	353	931
29	M	4	5 mg. % cho- line chloride	43	30.4		47	572	1,159
30	F	4	0.7% dl- methionine	41	11.8		8	115	341
31	F	5	None	18	34.6	9.1	25		1,423
32	F	5	None	21	46.3	8.3	27		1,403
33	F	5	10 mg. % cho- line chloride	60	48.7	8.4	20	629	1,358
34	F	5	25 mg. % cho- line chloride	60	43.2	8.7	20	562	1,098
35	F	5	50 mg. % cho- line chloride	60	25.9	8.0	9	114	316
36	F	5	100 mg. % cho- line chloride	60	16.9	9.6	9	72	239

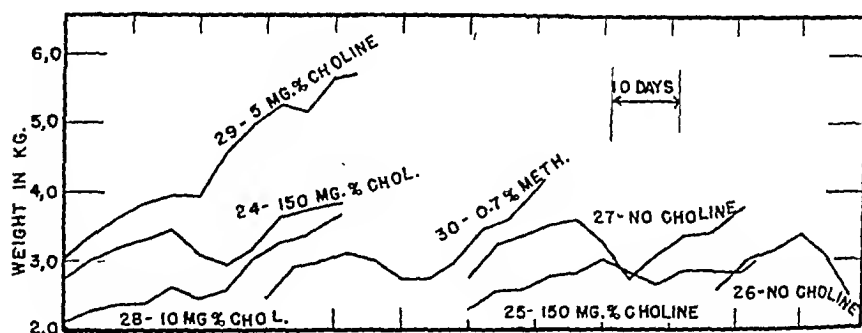


Fig. 5.—Growth curves of puppies of Experiment 5, Ration 4.

from the liver extract or to other lipotropic substances present in the liver extract cannot be stated. The liver feeding was stopped on the twenty-first day, however, and the experiment was continued until the fortieth day. At this time, liver function tests in the dogs receiving no choline and those receiving 5 mg. and 10 mg. per cent choline indicated considerable liver disease. All the dogs were sacrificed at this time. Terminal blood and tissue findings are given in Table III.

The failure of Ration 4 to provide good growth in all the control dogs when supplemented with 150 mg. per cent choline, coupled with the low plasma protein concentrations found in these animals, suggested that the ration was inadequate in protein. At this time the peanut oil meal became unavailable, and we were forced to obtain peanut protein from peanut flour. After ex-

III

IN PUPPIES IN EXPERIMENTS 5 AND 6

PRO- THROM- BIN TIME SEC.	PLASMA TOTAL CHOLE- STEROL MG. %	PLASMA CHOLE- STEROL ESTERS MG. %	BLOOD GLU- COSE MG. %	HEMO- GLOBIN GM. %	HEMA- TOCRIT %	PLASMA PRO- TEINS GM. %	BLOOD CREAT- ININE MG. %	MUSCLE "TOTAL" CREATINE MG. PER GM. FRESH	
								SKELETAL	HEART
11.5	97	67		11.5	37.5	3.9	1.10	3.50	
11.6	110	57		9.7	33.5	3.5	0.76	3.27	2.66
10.0				12.0	40.5	5.7	1.09	3.26	
13.2	60	30		11.8	39.0	4.2	0.78	3.35	
13.8	70	43		14.2	40.0	4.2	0.96	3.04	
18.0	83	22		11.5	39.0	3.6	0.78	3.48	2.52
10.0	90	60		11.6	32.5	4.8	1.14	3.48	
55.0	37	9		3.6	10.0	3.5	0.72	2.72	1.87
23.8	40	22	194	1.8	6.0	2.4	0.65	2.72	1.83
16.3	65	37	144	10.3	33.5	4.5	2.38	2.58	2.06
16.2	39	27	119	8.7	32.0	4.0	1.21	2.70	1.95
11.8	79	58	124	13.1	38.0	5.0	1.88	2.71	2.14
12.9	158	95	112	11.5	34.5	4.8	1.58	2.72	2.04

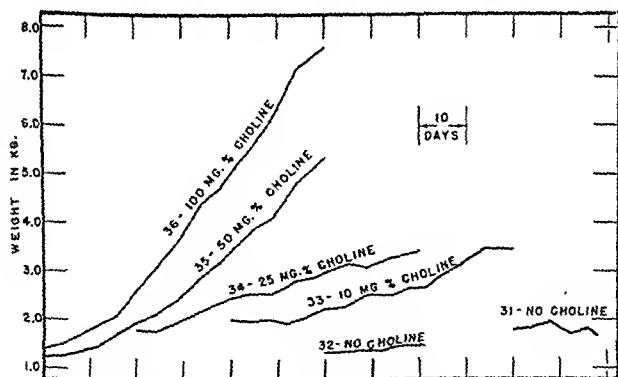


FIG. 5.—Growth curves of puppies of Experiment 5, Ration 5.

traction this peanut flour contains 64 per cent protein ($N \times 6.25$). It was decided to include the extracted peanut flour at a level of 15 per cent and to raise the casein content of the ration from 7 to 10 per cent. This change was expected to increase the value of the protein in the ration and would at the same time decrease the choline content of the ration. The composition of this ration is shown in Table I (Ration 5). A litter of six puppies was placed on

this ration (Experiment 6, Fig. 6). Two received the basal ration only, and the other four received the basal ration supplemented with 10, 25, 50, and 100 mg. of choline chloride per 100 Gm., respectively. Growth in Dog 36, receiving 100 mg. per cent choline, was excellent; fair in Dog 35, receiving 50 mg. per cent choline; and poor in Dogs 33 and 34, receiving 10 and 25 mg. per cent choline, respectively. Dogs 31 and 32, receiving no choline supplement, died on the eighteenth and twenty-first days, respectively. The onset of this fatal deficiency was rather sudden but was preceded by loss of appetite and activity several days before death. The animals were markedly undernourished, and there was pallor of the viscera. The liver was yellow, and the thymus gland was considerably smaller than normal. These observations are consistent with our findings in all of the dogs deficient in choline in Experiments 3, 4, and 5. Gross pathologic examinations were made by Dr. Frank Dutra, of the Department of Legal Medicine, and samples for histologic examination were taken by him; they will be discussed in a later publication. The remaining dogs in Experiment 6 were sacrificed on the sixtieth day for examination. Blood and tissue findings in this litter of dogs are summarized in Table III.

METHODS

The total lipid analysis of all liver samples was performed in the following way: The livers were dried in a constant temperature oven at from 62 to 65° C., ground in a small mortar, and extracted in a Soxhlet extractor for thirteen hours with chloroform. The per cent of chloroform-extractable material is given in terms of dry weight of liver throughout and is an average of two separate analyses.

Liver cholesterol was determined by the Liebermann-Burchard color reaction developed from a suitable aliquot of the chloroform solution of the liver lipids. The color of the extracts was corrected for by reading in the spectrophotometer at 710 μ before adding the sulfuric acid. Although this color may have changed somewhat by the addition of the acid, the error is necessarily small since these colors adsorb very little at 710 μ .

Blood plasma cholesterol and cholesterol esters were determined by the method of Bloor⁷ and Bloor and Knudson,⁸ respectively, and adapted to the spectrophotometer. Blood hemoglobin was determined by the acid hematin color measured in the spectrophotometer at 380 μ . Blood sugar was determined by the micromethod of Folin and Svedberg⁹ adapted to the spectrophotometer. Plasma proteins were determined by the plasma gravity method of Phillips and co-workers,¹⁰ using the equation:¹¹

$$(\text{Plasma Specific Gravity} - 1.00687) 340.1 = \text{Gm. plasma proteins per 100 c.c.}$$

The plasma protein determinations were frequently checked by the Kjeldahl method and favorable agreement found.

Blood creatinine was determined by development of the alkaline picrate color in tungstate filtrates of blood and measuring the color in the spectrophotometer at 500 μ . Muscle "total" creatine was determined by autoclaving finely cut muscle tissue in 2N sulfuric acid for forty-five minutes. The alkaline picrate color was developed from a tungstate filtrate of this preparation and measured in the spectrophotometer.

The bromsulfalein test was modified from the usual procedure in the clinic to one more suitable for work with dogs. Seven milligrams of bromsulfalein per kilogram of body weight were injected intravenously instead of the usual 2 to 5 mg., and the plasma samples were obtained after an interval of eight minutes. Normal adults and puppies have an almost colorless plasma after this time. This test is based on the quantity of dye present in the plasma after a given time (eight minutes) rather than the usual procedure of establishing the time required for the plasma to be completely decolorized. In practice all samples were obtained within plus or minus twenty seconds of the eight-minute mark by stop watch. The average deviation of all samples from eight minutes was thirteen seconds. Twelve-minute samples were obtained from several of the severely deficient dogs. It is interesting that these all contained over 80 per cent of the amount of dye present at eight minutes. Only dye concentrations of the eight-minute samples are listed in the tables. The determination of bromsulfalein in the plasma consisted of adding 4.5 c.c. distilled water and 1.0 c.c. normal NaOH in this order to 0.5 c.c. of the plasma in a spectrophotometer tube. The colors were immediately read in the spectrophotometer at the absorption maximum of 580μ . Since other substances in plasma absorb in highly varying amounts at this wave length, we found it necessary to use a "blank" for each dog made up with a sample of plasma taken before the dye injection.

The plasma phosphatase was determined by adding 1.0 c.c. of plasma from oxalated blood to 5 c.c. of 0.3 per cent sodium glycerophosphate adjusted to pH 7.6 with dilute HCl. Three tubes were used for each dog. One and one-half cubic centimeters of 10 per cent trichloroacetic acid were added to the first tube immediately; 5 drops of chloroform were then added to each tube, and the tubes were incubated at about 38° C. After eight hours, $1\frac{1}{2}$ c.c. of 10 per cent trichloroacetic acid were added to Tube 2 and after twenty-four hours, to Tube 3. The tubes were then centrifuged and suitable aliquots removed for inorganic phosphate analysis. This was done colorimetrically by the Fiske and Subbarow method, using the spectrophotometer at a wave length of 660μ . The zero hour phosphate was subtracted from the eight- and twenty-four-hour values in order to calculate the amount of inorganic phosphate liberated by the enzyme. No attempt is made to convert phosphorus values to enzyme units of any kind. Figures given are merely micrograms of phosphorus liberated by 1 c.c. of plasma in eight and twenty-four hours.

Prothrombin times were determined by rapid removal of jugular blood into a dry syringe and expressing 1 c.c. of this blood into a 9 mm. bore tube containing 0.2 c.c. of freshly prepared Winthrop's "Niphanoid" thromboplastin. The tube contents were mixed and the tube tipped back and forth until the clot formed. Two determinations were done with each dog. The method at best is crude and subject to limitations in interpretation, but after standardizing our technique, we were able to obtain excellent checks in control dogs. There was more variation in the deficient dogs. Prothrombin times are given in seconds from the time the 1 c.c. of blood came into contact with the thromboplastin. There is a few seconds' interval between the time the blood enters the syringe and is expressed into the tube, but our technique was uniformly rapid and this should not introduce a relative variable.

DISCUSSION

The failure of the control dogs receiving choline in Experiments 1 and 2 and the response of two of these animals to a liver fraction indicate that one or more substances essential for the nutrition of the dog were lacking on these rations. Since casein and methionine were ineffective in promoting growth in these animals, it was unlikely that amino acids were the sole deficiency factor. Improvement was afforded by using another liver extract (fraction "L") in Experiments 3, 4, 5, and 6. These results are surprising in view of past experience with purified rations for the dog. On purified rations supplemented only with thiamine, riboflavin, nicotinic acid, pyridoxine, pantothenic acid, and choline as sources of the vitamin B complex, fatal deficiencies were not observed over an experimental period of nine months. Only an occasional growth impairment or loss in weight¹² suggested that another factor was needed. Indeed an excellent ration for the production of pantothenic acid deficiency in the dog¹³ contained no natural vitamin B complex source whatever. This was a highly purified ration supplemented only with synthetic B vitamins. Growth was excellent in the control dogs receiving pantothenic acid, and there was little reason to suspect the necessity of an additional factor. However, other investigators have observed fatal deficiency in dogs receiving rations of this type over extended periods of time.¹⁴ Perhaps differences in the synthesis of nutrients by microorganisms of the gastrointestinal tract may account for these variations, although why synthesis might be different in the animals receiving the peanut meal ration is not known. It is also possible that the peanut meal exaggerates in some way the requirements for certain nutrients. Little can be said regarding the nature of the nutrient in Liver Fraction "L" essential in these experiments. It is probably not biotin, since the Fraction "L" is low in biotin and since a liver residue rich in biotin was found to be ineffective in preventing the death of Dogs 7 and 8 in Experiment 2. Fraction "L," however, is high in eluate factors essential for chicks and certain bacteria. The low plasma proteins in all the dogs of Experiment 5 (Table II) and the relatively poor growth of most of the control animals in Experiments 3, 4, and 5 (Figs. 3, 4, and 5) suggest, in addition, that the digestibility or quality of the peanut protein may be poor for the young pup. The data indicate that Rations 1 through 4 are not adequate for the weanling puppy, although choline deficiency may invariably be produced on them. However, Ration 5, which was used in Experiment 6, seems to meet all requirements, since excellent growth and higher plasma protein values were obtained in the control dog fed 100 mg. per cent choline, yet the ration was sufficiently deficient in choline to permit the development of a fatal deficiency in less than three weeks.

The growth and general condition of the methionine-fed dogs (20 and 30 in Experiments 4 and 5) indicate that this substance is effective in protecting the dog from choline deficiency. This is in accordance with the findings¹⁵ in rats. Thus the deficiency appears to be primarily of labile methyl groups and not of choline per se. There is, however, a small amount of choline in the ration since the liver extract (Fraction "L") contains approximately 0.9 mg. per gram, and presumably there are small amounts of choline phosphatides in the cottonseed oil and perhaps protein-bound choline still unextracted from the

peanut meal. Thus there remains the possibility of a specific requirement for choline *per se*, but, if existent, it is probably small in relation to the amount of labile methyl required.

Experiment 6 gives the clearest indication of the quantitative requirement for choline, since excellent growth was observed in the ration supplemented with 100 mg. per cent choline, and since this ration (5) contains the least amount of choline. It seems clear that 100 mg. of choline per 100 Gm. of ration was entirely adequate for Dog 36, since the rate of growth was optimum for laboratory dogs, and that 10 and 25 mg. per 100 Gm. of ration were inadequate for Dogs 33 and 34. Dog 35 showed a slightly fatty liver and growth somewhat inferior to that of Dog 36. Thus 50 mg. per cent appears somewhat inadequate on this ration, although the experiments are limited.

Food consumption studies show that the growing pup consumes from 50 to 60 Gm. of this ration per kilogram of body weight per day. The choline requirement on this ration must therefore be no more than 60 mg. of choline chloride per kilogram of body weight per day. This requirement is interesting in view of the requirement of the depancreatized dog. The amounts of choline and methionine in the ration used by Best and co-workers¹ were considerable, since it contained almost 75 per cent lean meat. These animals required, in addition, 1 Gm. or more of choline per day in order to prevent the fatty degeneration of the liver. Dragstedt¹⁶ also states that depancreatized dogs require "approximately 2.0 Gm. of choline a day over and above that present in the diet" to prevent the fatty metamorphosis of the liver. It should be noted that our experiments were all done with growing pups, and pups might be expected to require more choline than adult dogs. Reducing all these factors to a rough estimate, it would seem that a depancreatized dog might require over three times the amount of choline required by a normal dog. Considerable change in choline or labile methyl metabolism must therefore accompany pancreatectomy, and it is possible that other substances such as the "lipocaine" of Dragstedt,¹⁶ which are lipotropic but not choline and which are contained in raw pancreas, merely operate to restore the prepancreatectomy choline metabolism.

The rapidity with which choline deficiency appears under these conditions is somewhat comparable to that observed in rats¹⁷ and is a confirmation of the findings of Hough and associates.⁴ These investigators used a peanut meal ration similar to that described in this paper except that it was much higher in fat. They observed striking differences in hepatic dye clearance (rose bengal) and in serum phosphatase after their puppies had been on the ration only fourteen days. It is unfortunate that their animals developed infection and died before completion of the experiment, since further observations on the adequacy of their ration for the control animals would be most interesting in view of our experience with this type of ration.

Fonts³ reported a nutritional cirrhosis in dogs on a low choline ration which was isocalorically much higher in fat and lower in protein than the ration described in this paper. The protein was exclusively casein, so the methionine content was also greater than that of our rations, and liver fractions or other vitamin B complex sources were not used in most of the dogs. Adult dogs were used instead of puppies in most of the experiments, and the author states that there was a weight loss in all the dogs. The factors of age, lack of growth, and

higher methionine content of the ration point to a more chronic type of deficiency, as was actually the case. One group of dogs averaged 175 days survival on the ration.

The lipid content of the liver of the animals with choline deficiencies (Tables II and III) was extremely high and is comparable to that observed in the fatty infiltration of the liver of the puppy with pantothenic acid deficiency.¹³ The control range of from 7.4 to 20.5 per cent and the deficient range of from 25.9 to 56.6 per cent (dry weight basis) are quite distinct and all liver, with one exception, has been confined to these ranges. The exception was in the case of Dog 26 with choline deficiency in which the liver contained 19.5 per cent lipid. This animal was, as mentioned previously, sacrificed after the feeding of liver extract (Experiment 5), and this may have exerted lipotropic action due to its content of choline or other substances.

The total cholesterol values of the liver obtained in Experiment 6 (Table II) indicate that in spite of the rise of total lipids in the liver during the course of choline deficiency, the cholesterol content of the liver is relatively unaffected.

The elevation of the plasma phosphatase in the deficient dogs is clearly seen from Tables II and III. That of the choline-fed control animals ranged from 72 to 143 μg . phosphorus liberated per cubic centimeter of plasma in eight hours and from 222 to 399 μg . phosphorus in twenty-four hours. Typical values for dogs receiving no choline are 614 μg . phosphorus in eight hours, and 1,124 μg . in twenty-four hours (Dog 19). In general, the increase in phosphatase activity is related to the degree of fatty infiltration of the liver.

There is also clearly demonstrated a marked impairment in the removal of bromsulfalein from the plasma of the deficient dogs. The plasma from dogs receiving choline contained from 3 to 10 μg . of bromsulfalein per cubic centimeter of plasma after eight minutes, whereas dogs receiving no choline had from 12 to 42 μg . of the dye per cubic centimeter of plasma. However, the results of the bromsulfalein tests are not as well correlated with the degree of fatty infiltration of the liver as are the phosphatase values.

The prothrombin time increases were less striking, on the whole, and appeared only in those animals having the most severe fatty infiltration of the liver. Since all control animals were confined to the range 8.0 to 13.7 seconds, this must be regarded as "normal" under these conditions. Deficient Dogs 13, 26, 27, 28, and 29 all had prothrombin times in this range, and of these only one had a liver lipid content over 31 per cent. Much higher prothrombin time readings were observed in Dogs 12, 17, 18, 19, 31, and 32 (twenty-three to sixty-two seconds), and these animals had an average liver lipid content of 47.2 per cent.

The total cholesterol and cholesterol esters of the plasma in the control animals ranged from 90 to 158 and from 57 to 95 mg. per cent, respectively. The deficient animals ranged from 40 to 83 and from 9 to 58 mg. per cent, respectively. The average values for the control dogs are 114 mg. per cent total cholesterol and 70 mg. per cent cholesterol esters, while those for the deficient are 59 and 31 mg. per cent, respectively. Both cholesterol and cholesterol esters are therefore markedly reduced in the plasma of the animals deficient in choline, but the results indicate little correlation between the degree of fatty infiltration of the liver and the amount of lowering of either of the cholesterol components.

The changes in plasma phosphatase, cholesterol, and cholesterol ester values, bromsulfalein elimination, and prothrombin time are all commonly associated with liver disease. Most likely they do not reflect a primary biochemical effect of choline deficiency but rather a secondary effect incident to the fatty infiltration of the liver. The fall in hematocrit, hemoglobin, and plasma proteins observed in Dogs 31 and 32 and the rise in blood sugar in Dog 32 are doubtless other manifestations of this liver dysfunction. Evidently when a longer time is required for the liver to become infiltrated with fat, as in the case of Dogs 27, 28, 29, 33, and 34, the animal is able to maintain more normal concentrations of these substances.

The colloidal gold test as described by Gray¹⁸ was also applied to the sera of these dogs but revealed no differences whatever in the flocculating power of the sera between the dogs with severe liver impairment and those with normal liver findings. This was true in all ranges of sensitivity of the colloidal gold solution. As the colloidal gold test depends on the presence in the serum of increased amounts of gamma or beta globulin,¹⁹ it suggests that such increases do not occur in the pup with choline deficiency.

The dependence of creatine synthesis on the methyl "pool"²⁰ led us to investigate blood creatinine and muscle "total" creatine concentrations to see if there were any serious impairment in creatine formation. The skeletal muscle used throughout these studies was the pectoralis major. The two litters of dogs studied (Dogs 24 through 36, Table III) gave different "total" creatine concentration ranges. We are unable to explain these differences, but it can be seen from Table III that in either single experiment "total" creatine in both skeletal and heart muscle was not lowered in severe deficiency. This is in accordance with the findings of Almquist and co-workers²¹ in chicks and of Roberts and Eckstein²² in rats. There were no differences in the per cent dry weight of muscle tissue from dogs receiving choline and those receiving no choline, so the relationship of the creatine values holds in terms of both wet and dry muscle. The blood creatinine values are highly variable and difficult to correlate in any way with the state of nutrition of the animals. Whether or not total creatine excretion studies would indicate a decreased synthesis of creatine in the pups deficient in choline remains to be investigated. It is interesting that a tissue as rich in creatine as skeletal muscle can maintain a normal creatine content in the face of a severe methyl deficiency.

SUMMARY

The development of a ration for the production of acute choline deficiency in weanling puppies is described. Fatal deficiency in choline may be produced in less than three weeks and is characterized by severe fatty infiltration of the liver. Seven-tenths per cent dl-methionine or 0.1 per cent choline chloride supplemented to the ration apparently renders it adequate in all respects. In choline deficiency there is a rise in blood plasma phosphatase, an impairment in bromsulfalein elimination, and a fall in blood plasma cholesterol and cholesterol esters. In severe choline deficiency there is, in addition, an increase in prothrombin time and a decrease in blood hemoglobin, hematocrit, and plasma proteins. "Total" creatine in both heart and pectoralis major muscles is not lowered in choline deficiency, nor is there a consistent lowering of blood creatinine.

The total cholesterol concentration of the liver is unchanged, although total lipids are increased from three to four times.

The choline requirement of the growing puppy on this ration is probably not more than 100 mg. per 100 Gm. of ration or 50 mg. per kilogram of body weight per day.

We are indebted to Merek & Co., Inc., Rahway, N. J., for generous supplies of thiamine, riboflavin, nicotinic acid, pantothenic acid, pyridoxine, choline, and methionine; to The Wilson Laboratories, Chicago, Ill., for liver extracts; to Abbott Laboratories, North Chicago, Ill., for liver extrncts; and to Hynson, Westcott, and Dunning, Inc., Baltimore, Md., for bromsulfalein.

The authors wish to thank Mr. Fred Snell and Mr. Jose Sarraga-Audinot for their assistance in this work.

REFERENCES

1. Best, C. H., Ferguson, G. C., and Hershey, J. M.: Choline and Liver Fat in Diabetic Dogs, *J. Physiol.* 79: 94, 1933.
2. Schaefer, A. E., McKibbin, J. M., and Elvehjem, C. A.: Importance of Choline in Synthetic Rations for Dogs, *Proc. Soc. Exper. Biol. & Med.* 47: 365, 1941.
3. Fouts, P. J.: Vitamin B Complex Studies in Dogs: Production of Cirrhosis of Liver, *J. Nutrition* 25: 217, 1943.
4. Hough, V. H., Monahan, E. P., Li, T. W., and Freeman, S.: The Effect of Choline and Cystine on the Serum Phosphatase and Hepatic Dye Clearance of Dogs Maintained on Deficient Diets, *Am. J. Physiol.* 139: 642, 1943.
5. Engel, R. W., and Salmon, W. D.: Improved Diets for Nutritional and Pathologic Studies of Choline Deficiency in Young Rats, *J. Nutrition* 22: 109, 1941.
6. Hegsted, D. M., McKibbin, J. M., and Stare, F. J.: The Effect of Atabrine on Choline Deficiency in the Young Rat, *J. Nutrition* 27: 149, 1944.
7. Bloor, W. R.: The Determination of Cholesterol in Blood, *J. Biol. Chem.* 24: 227, 1916.
8. Bloor, W. R., and Knudson, A.: The Separate Determination of Cholesterol and Cholesterol Esters in Small Amounts of Blood, *J. Biol. Chem.* 27: 107, 1916.
9. Folin, O., and Svedberg, A.: Micro Methods for the Determination of Non-Protein Nitrogen, Urea, Uric Acid, and Sugar in Unclaked Blood, *J. Biol. Chem.* 88: 85, 1930.
10. Phillips, R. A., Van Slyke, D. D., Dole, V. P., Emerson, K., Jr., Hamilton, P. B., and Archibald, R. M.: Copper Sulfate Method for Measuring Specific Gravities of Whole Blood and Plasma, O.S.R.D. publication.
11. Weech, A. A., Reeves, E. B., and Goettsch, E.: The Relationship Between Specific Gravity and Protein Content in Plasma, Serum, and Transudate from Dogs, *J. Biol. Chem.* 113: 167, 1936.
12. Schaefer, A. E., McKibbin, J. M., and Elvehjem, C. A.: Studies on the Vitamin B Complex in the Nutrition of the Dog, *J. Nutrition* 23: 491, 1942.
13. Schaefer, A. E., McKibbin, J. M., and Elvehjem, C. A.: Pantothenic Acid Deficiency Studies in Dogs, *J. Biol. Chem.* 143: 321, 1942.
14. Lambooy, J. P., and Nasset, E. S.: The Inadequacy of Eight Synthetic B Vitamins for the Nutrition of Puppies—Unknown Factor (Factors) in Yeast and Probably Liver, *J. Nutrition* 26: 293, 1943.
15. Griffith, W. H.: Choline Metabolism. V. The Effect of Supplementary Choline, Methionine and Cystine and of Casein, Laetalbumin, Fibrin, Edestin and Gelatin in Hemorrhagic Degeneration in Young Rats, *J. Nutrition* 21: 291, 1941.
16. Dragstedt, L. R.: The Present Status of Lipocae, *J. A. M. A.* 114: 29, 1940.
17. Griffith, W. H., and Wade, N. J.: Choline Metabolism. I. The Occurrence and Prevention of Hemorrhagic Degeneration in Young Rats on a Low Choline Diet, *J. Biol. Chem.* 131: 567, 1939.
18. Gray, S. J.: The Colloidal Gold Reaction of Blood Serum in Diseases of the Liver, *Arch. Int. Med.* 65: 523, 1940.
19. Gray, S. J., and Barron, E. S. G.: The Electrophoretic Analyses of the Serum Proteins in Diseases of the Liver, *J. Clin. Investigation* 22: 191, 1943.
20. duVigneaud, V., Cohn, M., Chandler, J. P., Schenck, J. R., and Simmonds, S.: The Utilization of the Methyl Group of Methionine in the Biological Synthesis of Choline and Creatinine, *J. Biol. Chem.* 140: 625, 1941.
21. Almquist, H. J., Kratzer, F. H., and Meckel, E.: Further Experiments on Creatine Formation in the Chick, *J. Biol. Chem.* 148: 17, 1943.
22. Roberts, E., and Eckstein, H. C.: The Creatine Content of the Gastrocnemius Muscle of Young Male Rats on Diets Varying in Choline Content, *J. Biol. Chem.* 154: 377, 1944.

LYSOLECITHIN AND THE ANTIHEMOLYTIC VALUE OF THE BLOOD

H. BRUCE COLLIER, PH.D., AND KARL M. WILBUR, PH.D.
HALIFAX, N. S.

BERGENHEM and Fähræus¹ have found that when mammalian blood is incubated the erythrocytes tend to become spherical and their sedimentation velocity greatly reduced. Production of lysolecithin was believed to be responsible for these changes, and increased quantities of lysolecithin could be extracted from incubated serum, as compared with fresh serum. If the blood or serum were agitated during incubation, the changes did not take place. It was suggested by these authors that lysolecithin is formed by an enzymatic process in the relatively stagnant blood of the spleen, and that in the peripheral circulation the rapid movement of the blood prevents the formation of lysolecithin.

Singer² described an extraction method for the quantitative estimation of lysolecithin in serum; he confirmed the observation that lysolecithin increases on incubation of unmoved blood and that shaking inhibits the process. An increased production of lysolecithin in the stagnant blood from the splenic vein, and from varicose veins, was indicated. In a further investigation, Singer, Miller, and Dameshek³ found that splenectomy caused definite alterations in lysolecithin metabolism. Gripwall,⁴ using Singer's method, has confirmed most of these findings.

Nevertheless, it has not been conclusively proved that lysolecithin plays an important role in normal blood destruction, as Singer² admits. Furthermore, Gillespie⁵ has recently shown that the changes that take place in the erythrocytes of incubated blood are not the same as the changes produced by lysolecithin. Finally, it may be pointed out that Bergenhem and Fähræus¹ based their conclusions mainly upon measurement of sedimentation velocities, a method clearly not specific for lysolecithin. The reliability of Singer's extraction method has not been demonstrated, and in the present investigation it has been found that this method does not give quantitative recovery of added lysolecithin. In the absence of a specific quantitative method for determining lysolecithin in the blood, it was decided to adopt a direct titration method, which gives a measure of the net anti-hemolytic value of whole blood, or of serum, in terms of lysolecithin.

METHOD

The method employed is based upon the assumption that changes in the concentration of lysins, or of anti-hemolytic substances, in the blood will be reflected in the amount of lysolecithin required to bring about hemolysis of that blood under stated conditions. The procedure, a modification of that of Collier and Mack,⁶ consists of a determination of the number of milligrams of added lysolecithin which will bring about 50 per cent hemolysis of 1 c.c. of blood, or its equivalent, in 60 seconds at room temperature. This number is termed the

From the Departments of Biochemistry and Physiology, Dalhousie University.
Received for publication, June 16, 1944.

antihemolytic value. (The temperature coefficient of lysolceithin hemolysis was found to be very low—only 1.05 for the range 20 to 30° C.)

Apparatus.—A Lumetron Model 401 photoelectric colorimeter, with $\frac{1}{2}$ -inch tubes and red filter No. B660, was used for following hemolysis by measuring the opacity of the cell suspension as described by Wilbur and Collier.⁸

The hemolysis blank was negligible, and cell suspensions were found to obey Beer's Law, i.e., extinction or optical density ($-\log$ transmission) was proportional to cell concentration.⁹

Material.—The lysolceithin was the snake venom preparation of Collier and Allen⁷; although not pure, it provided a reproducible standard. It was dissolved in absolute methanol at a dilution of 1:1,000 for whole blood or serum and 1:4,000 for erythrocytes alone.

Buffered saline (4 volumes of 0.95 per cent NaCl plus 1 volume of M/8 sodium phosphate buffer, pH 7.4) was used for diluting the blood samples. Redistilled water was used for preparing solutions and for rinsing glassware.

Procedure.—For whole blood (anticoagulant 1:10,000 heparin†) 20 μ l. of the sample were diluted to 5 c.c. in the buffered saline. The suspension was stirred with a bulb pipette and at zero time the lysolceithin was added (about 50 to 80 μ l. of 1:1,000 solution from a pipette graduated in 1/1,000 c.c.). Fol-

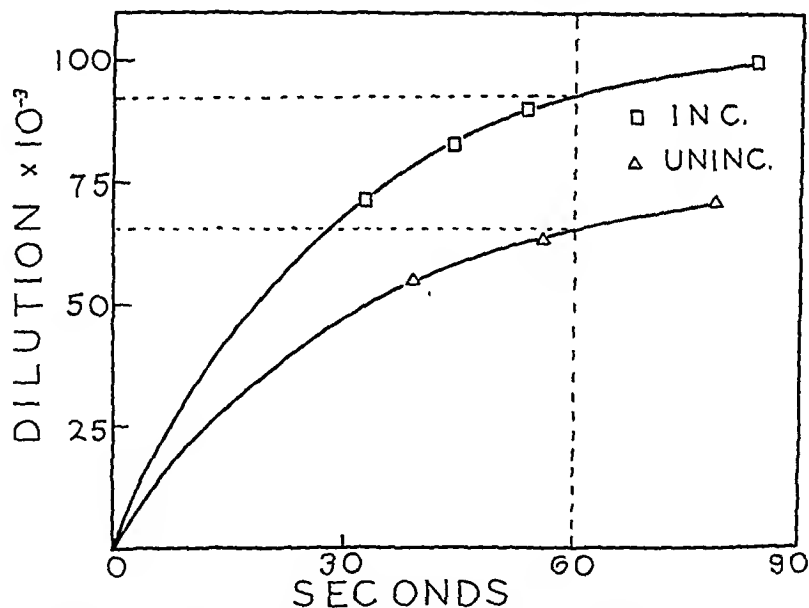


Fig. 1.—Lysolceithin time-dilution curves for human blood. Ordinates: lysolceithin dilution $\times 10^{-3}$ (e.g., 50 indicates a dilution of 1:50,000).

lowing immediate stirring the initial optical density was quickly noted and the colorimeter dial was set at 50 per cent of this reading. The time required for the galvanometer to read zero again was measured with a stopwatch, thus giving the time for 50 per cent hemolysis.

Several measurements were obtained for each sample, with hemolysis times somewhat less than or more than 60 seconds. From these values a conventional

*If a photoelectric colorimeter is not available, a visual colorimeter may be used for measuring opacity. The cell suspension is placed in one cup and a standard of opacity in the other. Under these conditions a longer time than 60 seconds for the end point is desirable.

†Connaught Laboratories.

time-dilution curve was plotted, with time of hemolysis as abscissae and lysolecithin dilution as ordinates. (For example, 50 μ l. of 1:1,000 lysolecithin in 5 c.c. = 1:100,000. Dilution = 100,000.) Typical time-dilution curves are given in Fig. 1.

From such a curve the dilution of lysolecithin required to bring about 50 per cent hemolysis in exactly 60 seconds is obtained by interpolation. Usually three points are sufficient and the precision of the method is about 2 to 3 per cent. From this dilution the antihemolytic value is calculated. For example, if the dilution corresponding to 60 seconds is 100,000, this is equivalent to 50 μ g of lysolecithin per 5 c.c. of 1:250 blood, or 2.5 mg. of lysolecithin per 1 c.c. of whole blood. The antihemolytic value is therefore 2.5

For erythrocytes alone the cells from 1 c.c. of blood were washed three times with the buffered saline and made up to 250 c.c. To 5 c.c. of this 1:250 suspension in the colorimeter was added 1:4,000 lysolecithin (about 50 to 100 μ l.), and the dilution required for 50 per cent hemolysis in 60 seconds was determined as before.

The antihemolytic value of plasma was obtained by deducting the value for the cells from that of whole blood. For serum, 20 μ l. were added to 5 c.c. of 1:250 cells of known antihemolytic value and the value for the cells alone was subtracted from that of the mixture. Type O cells were used for measuring a series of human untyped sera.

The antihemolytic value of blood reconstituted from washed cells and plasma was found to be identical with that of the whole blood, indicating that the use of washed cells is justified and that the antihemolytic value of plasma and of cells is additive. The sphering of cells induced by lysolecithin was not reversed by repeated washing.

The ratio of cells to plasma was obtained from hematocrit readings. In the case of incubated blood, the cells were so swollen that constant readings could not be made, and therefore plasma values could not be obtained directly.

When the Singer extraction method was applied to serum, the hemolysis readings were made photoelectrically in 1 c.c. microtubes, and the amount of lysolecithin in the extracts was determined by comparison with a hemolysis curve using standard lysolecithin and the same cells.

All incubations of blood or serum were carried out under sterile conditions.

RESULTS

Recovery of Lysolecithin by Singer's Method.—The Singer² extraction method was applied to both rabbit and human serum and the apparent increase in lysolecithin on incubation was confirmed. However, addition of lysolecithin to serum indicated that the recovery by this method was very low. When 0.20 mg. of lysolecithin was added to 10 c.c. of rabbit serum, none was recovered; addition of 10 mg. resulted in recovery of only 0.093 mg. Injection of 20 mg. of lysolecithin into a rabbit weighing 2.4 kg. caused no sphering of the cells and there was no increased recovery from the serum. This low recovery of added lysolecithin has been found to be due, in part, to incompleteness of the ether precipitation, apparent extraction of antihemolytic substances along with lysolecithin, and loss of activity of lysolecithin in saline solution.⁵

Finally, 10 mg. of lysolecithin were added to each 10 c.c. sample of human serum, incubated and uninincubated; an apparent increase of 0.038 mg. per cubic centimeter of serum was found (Table III). This procedure is not economical enough for routine use, but it probably indicates the order of magnitude of the increase in lysolecithin on incubation.

Incubation and the Antihemolytic Value of Blood.—The antihemolytic value of blood or serum should be lowered on incubation if the concentration of lysolecithin increases, other factors remaining unchanged. Table I indicates that when rabbit serum was incubated twenty-one hours at 37° C. there was a decrease in the antihemolytic value of much greater magnitude than could be accounted for by the increase in lysolecithin as determined by Singer's method. Table IV summarizes the results of experiments on whole blood, cells, and serum of three species, rabbits, dogs, and human beings, showing that the antihemolytic value is always significantly decreased by incubation. The influence of various factors upon this change in the antihemolytic value during incubation is described below.

TABLE I
INCUBATION AND THE ANTIHEMOLYTIC VALUE OF RABBIT SERUM

	LYSOLECITHIN (MG. PER C.C.)		
	UNINCUBATED	INCUBATED	DIFFERENCE
Singer's method	0.0005	0.0016	0.0011
Antihemolytic value	2.92	1.96	0.96

That the change in the antihemolytic value of the erythrocytes is due in part to an alteration in the plasma is indicated by the following procedure. Washed cells from 1 c.c. of rabbit blood were mixed with 2 c.c. of incubated serum, and another aliquot of cells was mixed with 2 c.c. of the same serum refrigerated. After two hours the cells were washed again and their antihemolytic value was measured. The values were 0.50 and 0.67, respectively. Assuming that lysolecithin is adsorbed by erythrocytes from incubated serum,¹ this corresponds to an adsorption of 0.085 mg. of lysolecithin from 1 c.c. of incubated serum.

The change in the antihemolytic value with *time* of incubation at 37° C. was measured on human serum from two individuals and the results are plotted in Fig. 2. Control values show that some change took place even at refrigerator temperature and that there was a further marked decrease in the antihemolytic value when these controls were finally heated for thirty minutes at 60 to 63° C. The maximum change took place in about twenty-four hours at 37° C. and subsequent incubations were carried out for this length of time.

To determine the effect of *temperature* upon the change in the antihemolytic value of rabbit serum, samples of the same serum were heated for eight hours at various temperatures. The results of these experiments are given in Fig. 3. The serum at 62° C. was cloudy, indicating incipient denaturation. No temperature optimum was observed, and it was repeatedly found that heating serum between 60 and 70° C. resulted in a very marked decrease in the antihemolytic value.

The effect of *H-ion concentration* was determined, using both human and rabbit serum. The pH was adjusted by addition of NaOH or H₃PO₄ to the test tubes before sterilization, and the serum was incubated twenty-four hours

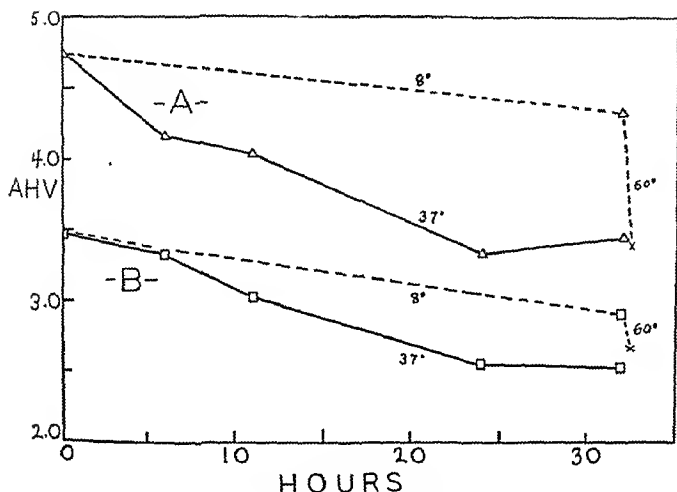


Fig. 2.—Change in the antihemolytic value on incubation of human serum.

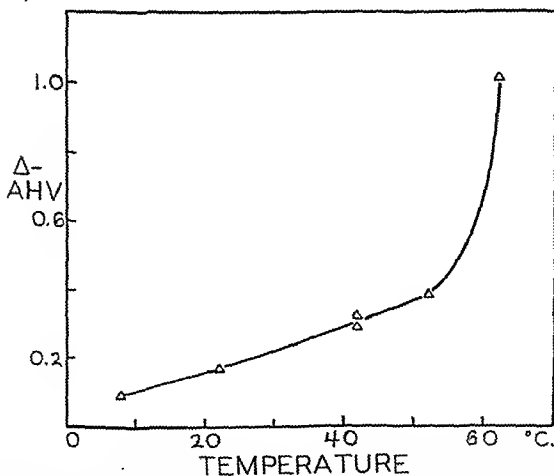


Fig. 3.—The effect of temperature on change in the antihemolytic value of rabbit serum.
Ordinates: Δ - antihemolytic value = decrease in the antihemolytic value on incubation.

at 37° C. (The tubes must be stoppered with rubber stoppers; cotton plugs permit loss of CO₂, resulting in an increase of pH.) The results obtained with one human serum and two rabbit sera are given in Fig. 4. It is apparent that there is an optimum at about pH 7.5.

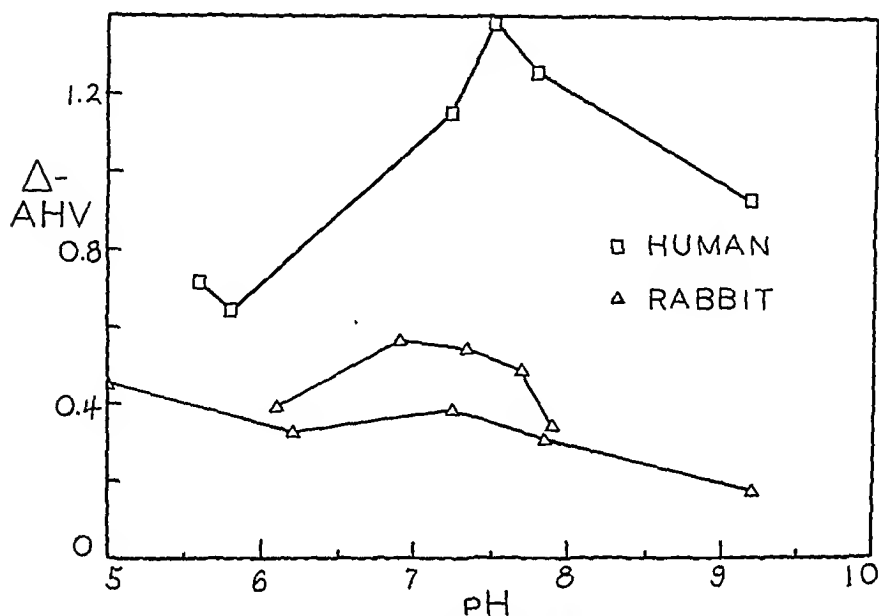


Fig. 4.—The effect of pH on change in the antihemolytic value of incubated serum. Ordinates: Δ - antihemolytic value = decrease in the antihemolytic value on incubation.

Shaking of serum or whole blood was carried out in L-shaped Pyrex rocker tubes, immersed in a constant temperature bath and rocked at fifteen oscillations per minute through an angle of 30 degrees. Control samples were simultaneously incubated at rest. The results of these experiments are summarized in Table II, from which it is seen that in four of six experiments rocking slightly diminished the change in the antihemolytic value.

TABLE II

THE EFFECT OF ROCKING ON THE ANTIHEMOLYTIC VALUE OF WHOLE BLOOD AND SERUM

	TEMP. (°C.)	DECREASE IN ANTIHEMOLYTIC VALUE		RATIO A/B
		(A) ROCKED	(B) AT REST	
Rabbit serum	20	0.40	0.40	1.00
Rabbit serum	37	0.36	0.44	0.82
Human whole blood	37	0.62	0.70	0.88
Human whole blood	37	0.54	0.67	0.80
Human whole blood	37	0.63	0.58	1.09
Human whole blood	37	0.70	0.73	0.96

Oxygen tension might be thought to have an effect on the changes taking place in stagnant blood, but experiments showed that reduction of the oxygen tension of whole blood had no marked effect on the decrease in the antihemolytic value. Samples of rabbit blood were evacuated, equilibrated with oxygen or nitrogen, and then incubated. It was found that the cells incubated under

N₂ were slightly more fragile and that a greater degree of spontaneous hemolysis had taken place. But the antihemolytic value of the whole blood showed no significant difference.

Various enzyme inhibitors were tested, but none of the following was found to have any effect on the change in the antihemolytic value of rabbit serum incubated twenty-four hours at 37° C.:

Quinine sulfate	1:5,000
Eserine sulfate	1:10,000
KCN	1:100,000
Tri-o-cresyl phosphate ^a	Saturated

Sodium fluoride, M/2,000, apparently accelerated the change in the antihemolytic value, but this may have been due to its hemolytic effect upon the erythrocytes. For this reason, fluoride is not a suitable anticoagulant for the measurement of the antihemolytic value. The oxalate mixture of Heller and Paul,¹⁰ used as an anticoagulant, gave results similar to those obtained with heparin, except that the cells were somewhat increased in fragility, even when kept in the refrigerator.

Chemical Changes in Incubated Serum.—The antihemolytic value of blood plasma is a resultant of various hemolytic and antihemolytic factors. Among the lytic substances present in plasma may be lysolecithin, bile salts, and free fatty acid.¹¹ According to Tsai and Lee,¹² free cholesterol is the chief antihemolytic agent. In order to assess the magnitude of the changes of some of these constituents, human serum was incubated and the following determinations were carried out. The Singer extraction method for lysolecithin was applied following addition of 10 mg. of lysolecithin to each 10 cc. of incubated and unincubated serum. Free cholesterol (i.e., digitonin-precipitable) was precipitated by Sperry's¹³ modification of the Schoenheimer-Sperry method, and the color was developed by the procedure of Rose, Schattner, and Exton,¹⁴ and the color was developed by the procedure of Rose, Schattner, and Exton,¹⁴ a method that gives a relatively intense and stable color. Free fatty acid was determined by the method of Freeman and Johnson,¹⁵ with electrometric titration in methanolic solution. The results of these analyses are given in Table III.

TABLE III
CHANGES IN CONSTITUENTS OF HUMAN SERUM ON INCUBATION

VALUES PER C.C. SERUM	UNINCUBATED	INCUBATED	DIFFERENCE	
			MG.	MICROEQUIV.
Antihemolytic value, as lysolecithin (mg.)	3.15	2.36	0.79	1.49*
Lysolecithin by Singer modified (mg.)	0.012	0.050	0.038	0.07*
Free cholesterol (mg.)	0.70	0.37	-0.33	-0.85
Free fatty acid (microequiv.)	2.20	1.45		-0.75

* Calculated as stearic lysolecithin.

It appears that the decrease in the antihemolytic value on incubation cannot be accounted for by the increase in extractable lysolecithin, and there is no increase in free fatty acid. The decrease in free cholesterol probably accounts for a considerable portion of the change in the antihemolytic value.

It was thought that chromatographic analysis might be applied to separation of the serum lipids and to the precise determination of lyso-

Preliminary experiments have indicated that lysolecithin is strongly adsorbed by alumina from CCl_4 or CHCl_3 solution and that it may be eluted with alcohol. Free cholesterol can be removed from the lipid extract by the method of Trappe.¹⁶ But when serum extracts were put through appropriate adsorption-elution procedures the recovery of added lysolecithin was still less than 1 per cent.

Summary of Antihemolytic Value Determinations.—Numerous antihemolytic value determinations have been carried out on the blood of rabbits, dogs, and human beings and the results have been summarized in Table IV. It is recommended, however, that each laboratory determine its own normal values rather than accept these figures as of absolute significance. (The antihemolytic value of blood specimens should be determined as soon as possible, as it decreases rapidly at room temperature and even in the refrigerator.)

TABLE IV
SUMMARY OF ANTIHEMOLYTIC VALUE DETERMINATIONS
(MEAN VALUES \pm STANDARD ERRORS)

	NO. OF SAMPLES	UNINCUBATED	INCUBATED	DIFFERENCE
<i>Rabbit whole blood</i>	4	2.45 ± 0.09	1.83 ± 0.12	0.62 ± 0.07
<i>Rabbit cells</i>	4	0.72 ± 0.07	0.52 ± 0.04	0.20 ± 0.03
<i>Rabbit serum</i>	13	2.20 ± 0.11	1.81 ± 0.11	0.39 ± 0.03
<i>Dog whole blood</i>	2	3.31 ± 0.41	1.76 ± 0.05	1.55 ± 0.35
<i>Dog cells</i>	1	0.80	0.45	0.35
<i>Dog serum</i>	2	2.59 ± 0.25	2.32 ± 0.13	0.27 ± 0.12
<i>Human whole blood</i>	7	3.20 ± 0.12	2.30 ± 0.08	0.90 ± 0.06
<i>Human cells</i>	7	0.68 ± 0.01	0.49 ± 0.01	0.19 ± 0.01
<i>Human serum</i>	4	3.97 ± 0.27	2.90 ± 0.16	1.07 ± 0.12
<i>Human serum*</i>	24	3.85 ± 0.08		

*From normal blood donors.

The series of human sera was obtained from normal blood donors. There was no significant difference between the averages for nine males and for fifteen females. Three of the sera were slightly lipemic and gave rather high values; one very lipemic specimen, with an antihemolytic value of 6.0, was not included in the average.

Saponin may also be used for antihemolytic value determinations but the distribution between cells and plasma is different from that obtained with lysolecithin. When saponin is used, the cells make up about two-thirds of the antihemolytic value of whole blood, while the ratio with lysolecithin is about one-fourth. With saponin the change in the antihemolytic value on incubation is relatively small: a sample of human blood gave the values 13.9 and 13.2 before and after incubation.

DISCUSSION

In confirmation of the findings of Bergenhem and Fåhraeus¹ and of Singer² it has been found that the apparent lysolecithin content of serum increases on incubation. Singer's method gives, however, less than 1 per cent recovery of added lysolecithin and attempts to achieve quantitative recovery have been unsuccessful. (It is possible that the lysolecithin formed by snake venom is not identical with that in the serum. When sufficient serum lysolecithin is available, its recovery by Singer's method will be tested.) Because of the

inadequacy of this method and because of the possibility that blood may contain various hemolytic and antihemolytic substances, a direct titration of whole blood with lysolecithin is proposed and the result is expressed as the antihemolytic value.

The capacity of the blood to withstand the effects of lysins obviously depends upon the resistance of both cells and plasma. Therefore any measure of this resistance to hemolysis must include both components. A quantitative measure of the total hemolytic and antihemolytic factors has been given in terms of lysolecithin. (Other lysins could be used but lysolecithin was chosen because it is known to be present in the blood. It cannot be assumed, of course, that the various lytic substances in the blood act in additive fashion, but the antihemolytic value in terms of lysolecithin is suggested as an approximation to the resistance of the blood to lysis by various agents *in vivo*.) The present method, like those mentioned above, fails to give an accurate measure of the amount of lysolecithin in blood, so that the question of its physiologic significance must still remain open.*

The antihemolytic value of blood or of serum has been found to decrease markedly on incubation. When whole blood is analyzed both plasma and cells show a decrease in the antihemolytic value. At least a portion of the change in the cells is due to changes in the plasma. However, as Gillespie⁸ has demonstrated, the volume and fragility changes that take place in the cells of incubated blood are not the same as those caused by lysolecithin.

Bergenheim and Fähræus¹ suggested that lysolecithin was produced in incubated blood through the action of a lecithinase, similar to that of snake venom.¹⁰ This enzyme in the blood was apparently completely inhibited by quinine, by shaking, or by heating to 52° C. The factors controlling the change in the antihemolytic value are evidently not identical with those that affect the sedimentation velocity, as shaking, lipase inhibitors, or heating did not inhibit the change. Furthermore, high temperatures accelerated rather than inhibited the change in the antihemolytic value, which fact suggests that the process is primarily nonenzymatic. The effect of pH is different from that observed by Bergenheim and Fähræus and more closely resembles the effect observed by Hughes¹⁷ with snake venom acting on lecithin films. Denaturation of serum protein or of lipoprotein complexes may be involved in the alteration in the antihemolytic value: higher temperatures and lower pH values, causing cloudiness of the serum, resulted in a marked lowering of the antihemolytic value (Figs. 3 and 4). On the other hand, addition of rattlesnake venom as a source of enzyme resulted in a decrease in the antihemolytic value greater than that brought about by incubation alone.

It is probable that when blood is withdrawn from the circulation, both enzymatic and nonenzymatic changes are initiated. These take place even at refrigerator temperatures and are accelerated at higher temperatures. The decrease in the antihemolytic value cannot be accounted for by the apparent increase in extractable lysolecithin. Johnson¹¹ has discussed the lytic action

*The values in Table III, together with the data on the decrease in the antihemolytic value of cells mixed with incubated serum, suggest that the increase in lysolecithin on incubation of serum is of the order of magnitude of 0.05 to 0.10 mg. per cubic centimeter. A concentration of 0.25 mg. per cubic centimeter was necessary for sphering of the erythrocytes of rabbit whole blood *in vitro*.

of free fatty acids, but these decrease on incubation, possibly because of esterification with cholesterol. Furthermore, lipemic human serum has a somewhat elevated antihemolytic value rather than a lowered value. A great part of the decrease in the antihemolytic value of incubated serum may be due to the reduction in free cholesterol, a phenomenon which was described by Sperry and Stoyanoff.¹⁸ If it is assumed that free cholesterol inactivates lysolecithin in molecular proportion,¹⁹ more than one-half of the change in the antihemolytic value of human serum can be accounted for in this way (Table III).

The experiments on the antihemolytic value of blood offer no support for the "stagnation" hypothesis of normal hemolysis, since neither shaking nor reduction of oxygen tension had any marked effect on the antihemolytic value. Certain mechanisms must operate to maintain the antihemolytic value of the blood at a fairly constant level in vivo, but these evidently fail to operate in vitro. The practical significance of the antihemolytic value has not as yet been demonstrated, but it is planned to investigate the effect of various physiologic and pathologic conditions on the antihemolytic value of blood.

SUMMARY

1. It has been confirmed that extractable lysolecithin increases on incubation of serum. The Singer extraction method gives, however, less than 1 per cent recovery of added lysolecithin.

2. In the absence of a specific method for lysolecithin, the antihemolytic value of whole blood, erythrocytes, and serum has been determined by titration with lysolecithin. The antihemolytic value is defined as the number of milligrams of lysolecithin required to bring about 50 per cent hemolysis of 1 c.c. of blood, or its equivalent, in one minute at room temperature. Average values are presented for antihemolytic value determinations on blood of rabbits, dogs, and human beings.

3. The antihemolytic value of whole blood or serum decreases markedly on incubation. A rise in temperature accelerates the change, with no evidence of a temperature optimum. The pH optimum is at about pH 7.5.

4. The change in the antihemolytic value of incubated serum is not markedly affected by agitation, reduction in oxygen tension, or lipase inhibitors.

5. This change in the antihemolytic value may be partly due to the decrease in free cholesterol on incubation. It is probably the resultant of various changes in the serum or plasma proteins and lipids. It is not known whether this phenomenon has any physiologic significance.

We wish to acknowledge the kind cooperation of the staff of the Halifax Blood Donors' Clinic in obtaining small samples of human serum.

NOTE.—Attention should be drawn to a recent paper which appeared after this paper had been submitted for publication: Ponder, E.: The Kinetics of in vivo Hemolytic Systems, *J. Gen. Physiol.* 27: 483, 1944.

REFERENCES

1. Bergenhem, B., and Fåhræus, R.: Ueber spontane Hämolsinbildung im Blut, unter besonderer Berücksichtigung der Physiologie der Milz, *Ztschr. f. d. ges. exper. Med.* 97: 555, 1936.
2. Singer, K.: Lysolecithin and Hemolytic Anemia. The Significance of Lysolecithin Production in the Differentiation of Circulating and Stagnant Blood, *J. Clin. Investigation* 20: 153, 1941.

3. Singer, K., Miller, E. B., and Dameshek, W.: Hematological Changes Following Splenectomy in Man, *Am. J. M. Sc.* 202: 171, 1941.
4. Gripwall, E.: Zur Klinik und Pathologie des hereditären hämolytischen Ikterus mit des Verhaltens der roten Blutkörperchen, *Acta med.*
5. Gille, . of Lysolecithin and of Incubation on the Shape, Size, and Fragility of Erythrocytes, *Quart. J. Exper. Physiol.* 32: 113, 1943.
6. Collier, H. B., and Mack, G. E., Jr.: Vitamin B and Phenothiazine Anæmia in Dogs, *Canad. J. Research, Sect. E* 22: 1, 1944.
7. Collier, H. B., and Allen, D. E.: The Hemolytic Action of Phenothiazine Derivatives, *Canad. J. Research, Sect. D* 20: 283, 1942.
8. Wilbur, K. M., and Collier, H. B.: A Comparison of the Hemolytic Actions of Lysolecithin and Saponin, *J. Cell. & Comp. Physiol.* 22: 233, 1943.
9. Hottinger, A., and Bloch, H.: Ueber die Spezifität der Cholin-esterase-Hemmung durch Tri-o-kresyl-phosphint, *Helv. Chim. Acta* 26: 142, 1943.
10. Heller, V. G., and Paul, H.: Changes in Cell Volume Produced by Varying Concentrations of Different Anticoagulants, *J. Lab. & Clin. Med.* 19: 777, 1934.
11. Freeman, L. W., Loewy, A., and Johnson, V.: In Vivo Hemolysis Produced by Soap Injection, *Am. J. Physiol.* 140: 556, 1944.
12. Tsai, C., and Lee, J. S.: The Nature of Authemolytic Substances in the Plasma, *Chinese J. Physiol.* 16: 165, 1941.
13. Sperry, W. M.: The Schoenheimer-Sperry Method for the Determination of Cholesterol, New York, 1943 (mimeographed).
14. Rose, A. R., Schattner, F., and Exton, W. G.: A Method for Determining Blood Cholesterol, *Am. J. Clin. Path.* 11: 19, 1941.
15. Freeman, L. W., and Johnson, V.: The Hemolytic Action of Chyle, *Am. J. Physiol.* 130: 723, 1940.
16. Trappe, W.: Eine einfache Methode zur getrennten, quantitativen Bestimmung von freiem und verestertem Cholesterin im Blutserum ohne Digitoninfällung und Verseifung, *Ztschr. f. physiol. Chem.* 273: 177, 1942; *Chem. Abstr.* 37: 3119, 1943.
17. Hughes, A.: The Action of Snake Venoms on Surface Films, *Biochem. J.* 29: 437, 1935.
18. Sperry, W. M., and Stoyanoff, V. A.: The Enzymatic Synthesis and Hydrolysis of Cholesterol Esters in Blood Serum, *J. Biol. Chem.* 126: 77, 1938.
19. Delzenne, C., and Fournau, E.: Constitution du Phosphatide Hémolysant (Lysoécithine) Provenant de l'Action du Venin de Cobra sur le Vitellus de l'Oeuf de Poule, *Bull. Soc. Chim.* [4] 15: 421, 1914.

THE EFFECT OF HUMIDITY AND TEMPERATURE ON OXYGEN TOXICITY

H. R. HULPIEU, PH.D., AND VERSA V. COLE, PH.D., M.D.
INDIANAPOLIS, IND.

INTRODUCTION

TOXICITY from oxygen has been repeatedly demonstrated.¹⁻⁶ The toxicity is dependent on the concentration of oxygen present and varies with the species.^{2, 6, 7} In human beings concentrations as low as 70 per cent for a time as short as six days have been reported to cause oxygen toxicity.⁸ In some species of animals, such as the rat, it also varies with age.^{9, 10} In other species, such as the dog, no such age difference was found.¹¹ There is also evidence that oxygen toxicity is increased by increasing the external temperature. Thus Campbell showed that rats taken to six atmospheres of oxygen and slowly decompressed had a higher survival rate at 24° C. than at 33° C.¹² Turtles brought to mammalian temperature (37.5° C.) behaved like mammals, whereas at 23 to 26° C. they showed no symptoms.³ The work of Paine and others showed that a dog whose external environment went to 90° F. did not survive as long as the other dogs which were maintained at a lower temperature.¹¹ The humidity has been recorded or kept constant in several studies on oxygen toxicity,^{6, 7, 9-11, 13} but there seems to be a dearth of comparisons on the effect of humidity. It was our object, therefore, to study further the effects of temperature and humidity on oxygen toxicity.

EXPERIMENTAL PROCEDURE

A diagram of the apparatus used may be seen in Fig. 1. The chamber consisted of a 5-gallon glass jar with inlet and outlet tubes. The inlet tube was connected with two bottles which contained either water or calcium chloride, depending on the humidity desired. These connected directly with the oxygen tank or the outside air. Rats were placed in a wire cage within the chamber, four rats to a chamber (two on each side), at humidities 30 per cent and 90 per cent or higher. Two rats were used per chamber for humidities below 20 per cent. For the 30 per cent humidity, calcium chloride was placed in the stand holding the cage. For the lowest humidity it was necessary to surround the cage with a screen containing calcium chloride in addition to that in the stand. For humidity near saturation, water was placed about 1 cm. deep in the bottom of the chamber. A thermometer with a relative humidity measuring device was attached to the rat cage. These instruments were checked against wet and dry bulb thermometers and found to be accurate from about 10 to 40 per cent humidity. The high humidity experiments were

From the Department of Biochemistry and Pharmacology, Indiana University School of Medicine.

Received for publication, July 27, 1944.

checked at intervals with the wet and dry bulb thermometers and also by Harger's glass syringe apparatus for determining moisture in air.¹⁴

Rats were selected as experimental animals because of their well-known resistance to oxygen poisoning.^{9, 10, 15} Male rats were used throughout. The rats varied in weight from 69 to 111 Gm.; the average weight for the various groups varied from 79 to 91 Gm. The temperatures used for the experiment were 90 and 80° F., with variations of plus or minus 2 degrees. The humidities studied were a low humidity between 10 and 20 per cent, an intermediate humidity averaging about 30 per cent with maximum variations from 25 to 35 per cent, and a high humidity of 90 per cent or above. Analysis for carbon

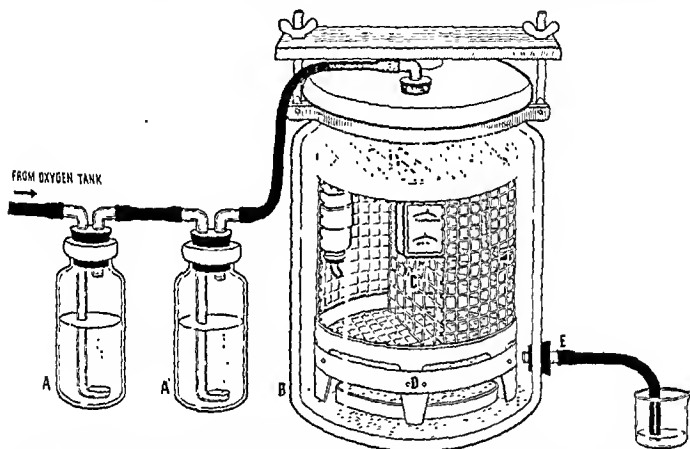


Fig. 1.—Diagram of apparatus used. A and A', 500 ml. bottles for humidifying or dehydrating air or oxygen; B, 5-gallon glass jar with air-tight cover; C, divided wire cage with thermometer and humidity recorder attached; D, wire stand for CaCl_2 ; E, outlet tube.

dioxide by the Haldane apparatus showed the concentration to be kept below $2\frac{1}{2}$ per cent. This was done by varying the stream of oxygen or air. This concentration of carbon dioxide is well below that which might produce toxic effects.¹⁶ Analysis for oxygen showed it to be above 96 per cent in all oxygen experiments.

Each experiment ran for ten consecutive days or until death of all the rats. On each day the rats were removed from their cages for twenty minutes to allow for weighing of the animals and cleaning of the cages. At the end of the ten days the rats were removed from the chambers and kept in the laboratory for at least two more days to see if any died. All that died were autopsied and checked for pathologic changes of oxygen poisoning.

RESULTS AND DISCUSSION

For the experiments on air, ten to twelve rats were used for each temperature and humidity. None of the rats died. The gain in weight per

(Fig. 2) was nearly normal with the exception of that for the rats kept at 90° F. and below 20 per cent humidity. There seems, then, to be nothing in the procedure which would injure the health of the rats with the possible exception of the combined high temperature and low humidity in one experiment.

In Table I and Fig. 3 the results of high oxygen concentration at two temperatures and three humidities are recorded. It can be seen from these data that oxygen had an unfavorable effect on both weight and life of all the groups of animals studied. Our observations confirm those of previous workers, that a high temperature is unfavorable.^{11, 12} This can be seen from the number surviving at each of the humidities studied. At humidity 90 per cent there is a chance of 1 in 100,000 that the difference between 80 and 90° F. is not significant. At humidity 30 per cent the chance is less than 1 in 1,000,000 that

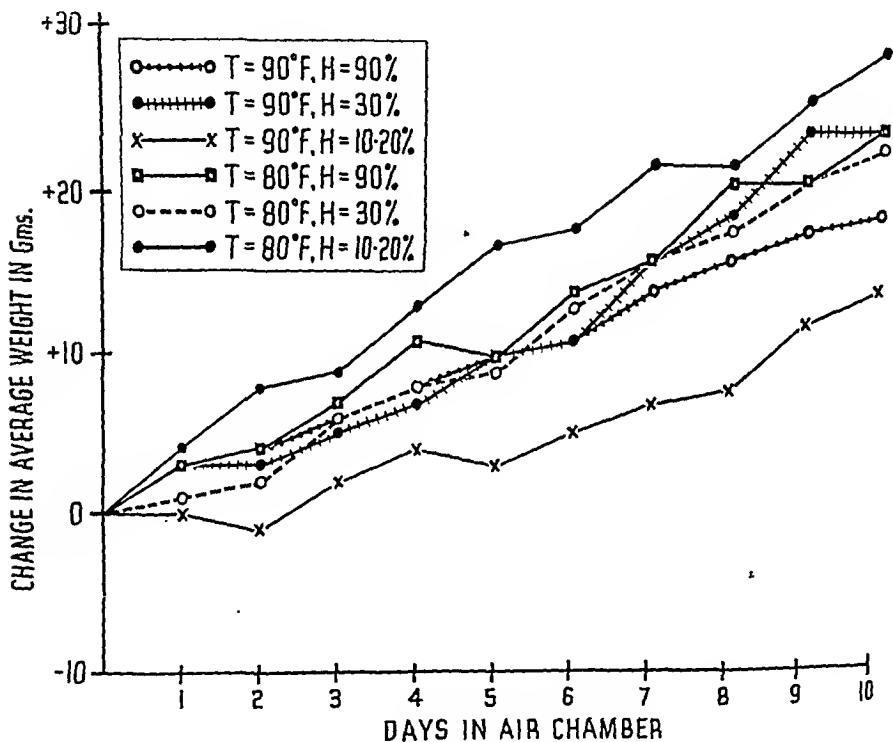


Fig. 2.—Effect of temperature and humidity on weight. The rats were kept in the chamber illustrated in Fig. 1 but in air instead of oxygen.

the difference is not significant. At humidity 20 per cent the chance that the difference is not significant is less than 1 in 100. If the best survival rate at temperature 90° F. is compared with the worst at temperature 80° F., it can be seen that the chance is very slight that there is any difference between these two groups. Temperature 80° F. and humidity 30 per cent gave the best results in the six groups studied. The chance that this does not differ from temperature 80° F. and humidity 90 per cent is less than 1 in 100. The chance that humidity 30 per cent does not differ from humidity less than 20 per cent is about 1 in 10,000. At ten days there is no evidence that the difference between 20 per cent humidity and 90 per cent at 80° F. is significant. However,

TABLE I

THE EFFECT OF OXYGEN ON THE SURVIVAL AND WEIGHT OF RATS

DAYS IN OXYGEN	T = 80° F. H = 90%		T = 80° F. H = 30%		T = 80° F. H = <20%		T = 90° F. H = 90%		T = 90° F. H = 30%		T = 90° F. H = <20%	
	NO. OF RATS LIVING	AV. WT.	NO. OF RATS LIVING	AV. WT.	NO. OF RATS LIVING	AV. WT.	NO. OF RATS LIVING	AV. WT.	NO. OF RATS LIVING	AV. WT.	NO. OF RATS LIVING	AV. WT.
0	23	85	28	85	14	82	16	89	24	88	16	85
1	23	83	28	87	14	86	14	89	24	87	14	79
2	22	78	28	84	13	79	7	75	21	81	6	73
3	21	74	28	78	9	74	2	75	10	78	0	78
4	19	70	28	75	5	70	2	67	9	78	—	—
5	17	71	28	76	5	72	2	66	9	80	—	—
6	17	71	27	81	5	71	1	66	6	80	—	—
7	16	74	27	80	5	71	1	78	6	82	—	—
8	16	74	27	80	5	71	1	80	6	81	—	—
9	16	73	27	79	5	71	1		6	78	—	—
10	16	73	27	79	5	70	1		6	80	—	—
2 days after removal	14		26		5		1		6			

The average weight applies not only to those living, but also to the rats that died in the preceding twenty-four hours.

T, Temperature; H, Humidity.

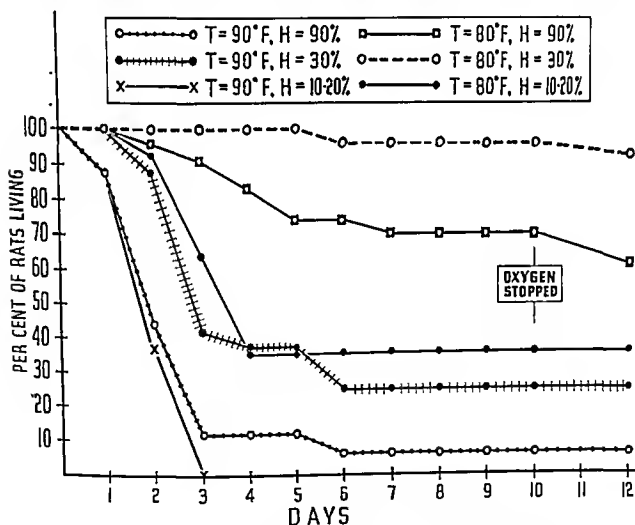


Fig. 3.—Survival of rats in an atmosphere of 96+ per cent oxygen for ten days.

if a comparison is made of the number dead on the fourth day, the chances are about 1 in 300 that the difference is not significant. This would seem to indicate that there is a difference in death rate although not a significant difference in total deaths over a period of ten days. For temperature 90° F. the best results were also obtained at 30 per cent humidity. Chances are about

1 in 200 that the humidity below 20 per cent is not worse than at 30 per cent. The chances that humidity 90 per cent is not worse than humidity 30 per cent are about 1 in 15.

CONCLUSIONS

A temperature of 90° F. is unfavorable for rats in high concentrations of oxygen when compared with a temperature of 80° F. at the three relative humidities studied. Humidity of 30 per cent is more favorable than humidities below 20 per cent or above 90 per cent. In the control of oxygen toxicity both the temperature and humidity are important.

REFERENCES

1. Smith, J. L.: The Pathological Effects Due to Increase of Oxygen Tension in the Air Breathed, *J. Physiol.* 24: 19, 1899.
2. Binger, C. A. L., Faulkner, J. M., and Moore, R. L.: Oxygen Poisoning in Mammals, *J. Exper. Med.* 45: 849, 1927.
3. Faulkner, J. M., and Binger, C. A. L.: Oxygen Poisoning in Cold Blooded Animals, *J. Exper. Med.* 45: 865, 1927.
4. Kaunitz, J.: Myocardial Damage Resulting From High Oxygen Tension, *J. Aviation Med.* 13: 267, 1942.
5. Piehotka, J.: Ueber die histologischen Veränderungen der Lunge nach Atmung von hochkonzentriertem Sauerstoff im Experiment, *Beitr. z. path. Anat. u. z. allg. Path.* 105: 381, 1941.
6. Liebegott, G.: Ueber Organveränderungen bei langer Einwirkung von Sauerstoff mit erhöhtem Partialdruck im Tierexperiment, *Beitr. z. path. Anat. u. z. allg. Path.* 105: 413, 1941.
7. Clamann, H. G., and Becker-Freyseng, H.: Einwirkung des Sauerstoffs auf den Organismus bei Hoherem als normalem Partialdruck unter besonderer Berücksichtigung des Menschen, *Luftfahrtmedizin* 4: 1, 1940.
8. Moody, E., and Howard, W. M.: Probable Oxygen Poisoning Produced in an Ordinary Oxygen Tent, *Arch. Pediat.* 59: 458, 1942.
9. Smith, F. J. C., Heim, J. W., Thomson, R. M., and Drinker, C. K.: Body Changes and Development of Pulmonary Resistance in Rats Living Under Compressed Air Condition, *J. Exper. Med.* 56: 63, 1932.
10. Smith, F. J. C., Bennett, G. A., Heim, F. W., Thomson, R. M., and Drinker, C. K.: Morphological Changes in the Lungs of Rats Living Under Compressed Air Conditions, *J. Exper. Med.* 56: 79, 1932.
11. Paine, J. R., Lynn, D., and Keys, A.: Observations on the Effects of the Prolonged Administration of High Oxygen Concentrations to Dogs, *J. Thoracic Surg.* 11: 151, 1941.
12. Campbell, J. A.: Body Temperature and Oxygen Poisoning, *J. Physiol.* 89: 17P, 1937.
13. Becker-Freyseng, H., and Clamann, H. G.: Zur Frage der Sauerstoffvergiftung, *Klin. Wchnschr.* 18: 1382, 1939.
14. Harger, R. N.: To be published.
15. Boycott, A. E., and Oakley, C. L.: Oxygen Poisoning in Rats, *J. Path. & Bact.* 35: 468, 1932.
16. Barbour, J. H., and Seever, M. H.: Narcosis Induced by Carbon Dioxide at Low Environmental Temperatures, *J. Pharmacol. & Exper. Therap.* 78: 296, 1943.

THE VARIABILITY OF HEART RATE AND BLOOD PRESSURE IN SELECTED GROUPS OF COLLEGE AND HIGH SCHOOL STUDENTS

ELIZABETH POWELL SALIT, PH.D., AND W. W. TUTTLE, PH.D.
IOWA CITY, IOWA

IF CARDIOVASCULAR measures are to be used to identify persons in good and poor physical condition or to measure progress during the course of a training program, these measures must have sufficient reliability and discriminatory power. Individuals are known to differ from each other as to resting and postexercise heart rate and blood pressure. It is also known that each individual varies from day to day and during the course of the day. In order to be able to distinguish one individual or group from another, it is necessary to have a comparatively wide range of variability among individuals or groups. Furthermore, repeated measures on the same individuals should show relatively small fluctuations. Otherwise the obtained differences between individuals could very well be due to chance variations in the individuals concerned or to errors in measurement. The measurable effects of a training program or medically prescribed treatments are likely to be quite small. For this reason any objective measure of progress should be highly reliable, that is, not subject to large chance variations. It is the purpose of this investigation to determine which of several commonly used cardiovascular measures are most reliable and which of them have the greatest discriminatory power.

Procedure.—Two groups of data were collected. The first was obtained from twenty college men and forty college women. One-half of the men and one-half of the women participated regularly in vigorous physical activity. The others reported little or no vigorous exercise. The second group of data was obtained from five 15-year-old high school boys who had an initial resting pulse between 70 and 80 beats per minute.¹

Each of the college subjects came to the laboratory on four different days, usually at weekly intervals and at the same time of day. After five minutes of bed rest the pulse was counted for fifteen seconds, and the blood pressure (systolic and diastolic) was determined according to standard clinical procedures. The resting pulse and blood pressure determinations were repeated until consecutive readings were approximately the same. In only a few cases was it necessary to take a third set of readings, indicating that five minutes of bed rest were usually sufficient to stabilize the pulse and blood pressure at a resting level. The subjects then rode a stationary bicycle for one minute at moderate speed (60 revolutions per minute). A one-half-minute interval was allowed for resuming the reclining position and adjusting the cuff of the sphygmomanometer. Reclining pulse and blood pressure determinations were made one-half minute after the exercise was completed and again after two and four minutes had elapsed. This whole procedure was repeated on different occasions until four sets of readings were recorded for each subject.

From the Department of Physiology, State University of Iowa.
Received for publication, April 10, 1944.

In the case of the high school boys, heart rates were recorded each school day morning during a period of five months. The sitting pulse was counted before and after a standard stool stepping exercise (40 steps per minute for one minute, using a thirteen-inch stool). The postexercise pulse was counted continuously for two minutes, starting immediately after the cessation of the exercise.

Individual Means and Variability.—The original scores for the resting heart rate, the individual means arranged according to resting heart rate, and the averages for the vigorously and moderately active groups of college men and women are shown in Fig. 1. The corresponding data for the pulse one-half minute after exercise and for the increase due to exercise appear in the same order in Figs. 2 and 3. It is evident by inspection that the range of scores for

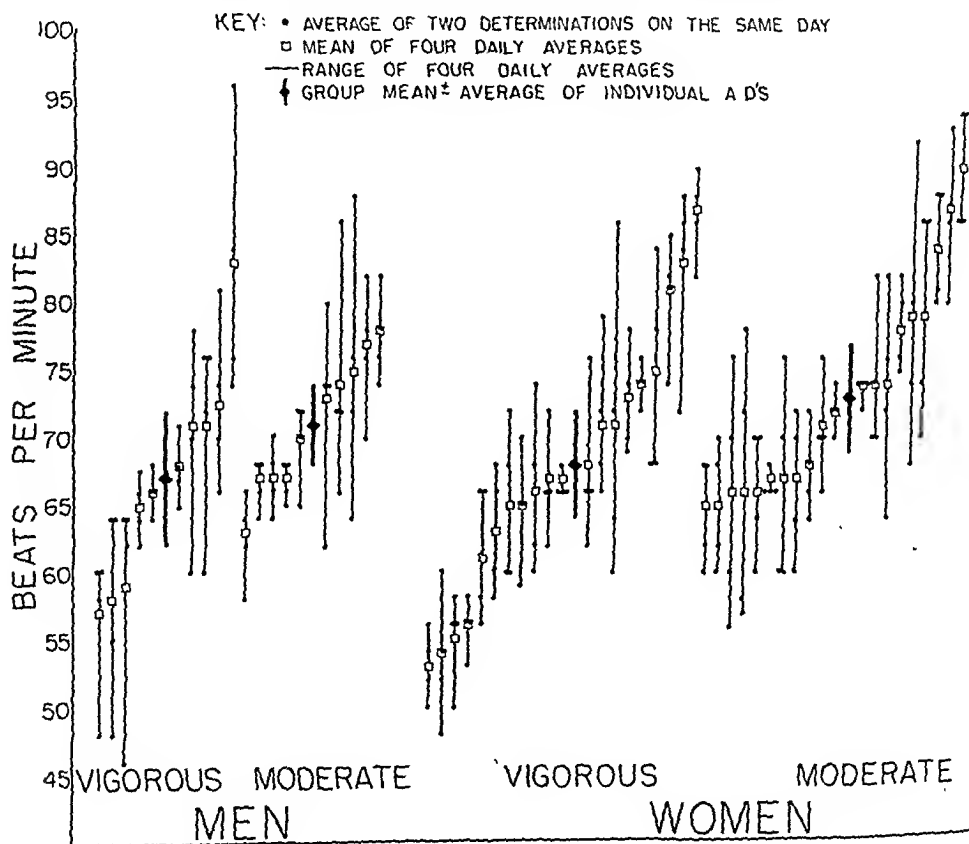


Fig. 1.—Resting heart rate for vigorously and moderately active groups of college men and women.

individuals covers a large part of the range for the group; also, that the rank order established in Fig. 1 does not hold for the postexercise pulse or for the increase due to exercise, that is, the correlation with resting heart rate is not very high.

High, low, and average values for the average deviation of single measures from the means for individuals are shown in Table I. The men and women are about equally variable, averaging from 4 to 5 beats per minute in heart rate and

from 3 to 5 mm. of mercury in blood pressure. The day-to-day average deviations in the heart rate of the 15-year-old boys ranged from 2 to 7 beats per minute and yielded averages which were about the same as those for the college groups.

The average deviation from the mean includes 57 per cent of the scores in a normal distribution, whereas 43 per cent of the scores fall beyond the limits set by the average deviation. Thus if a person's average resting heart rate was

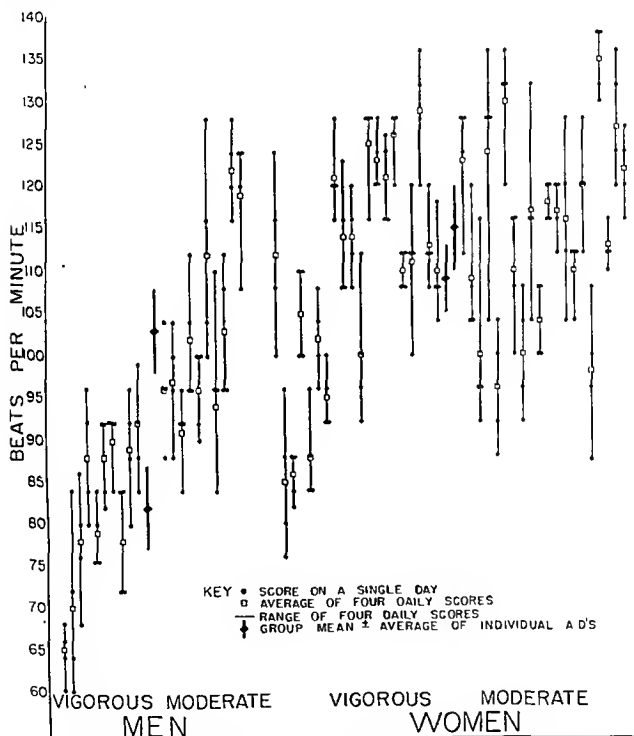


Fig. 2.—Heart rate one-half minute after moderate exercise. (Subjects arranged as in Fig. 1.)

75 beats per minute and his average deviation was 6 beats, the chances are that about three-fifths of any subsequent scores would be between 69 and 81 beats per minute. About one-fifth of the scores would be greater than 81 and another fifth would be less than 69. It would seem that fluctuations of this magnitude are altogether too large to warrant the use of single scores as measures of physical condition.

be certain at the 1 per cent level of confidence that the difference is not due to chance. Differences of 11 beats per minute are significant at the 5 per cent level of confidence.

Differences as large as 17 beats per minute in the resting pulse occurred only 15 times out of a possible 190 in our group of twenty men. Thus the differences in resting pulse would be significant at the 1 per cent level of confidence only 8 per cent of the time. Differences of 11 beats occurred 52 times (27 per cent of the time). Ten of these differences (5 per cent of 190) would have been significant on the basis of chance alone. The remaining forty-two cases represent the efficiency of the resting pulse in discriminating at the 5 per cent level among individuals in our group of twenty college men.

The pulse differences required for men and women at the 1 per cent and the 5 per cent level, the number of times differences as large as these occurred, and the percentage of differences meeting the 1 per cent and the 5 per cent requirement are shown in Table III. The greatest discriminatory power is shown by the pulse one-half minute after exercise for men. Of the 190 differences between individuals in this group, 77 (41 per cent) were large enough to be significant at the 1 per cent level of confidence and 117 (62 per cent) were large enough to be significant at the 5 per cent level of confidence.

TABLE III

THE OCCURRENCE OF HEART RATE DIFFERENCES LARGE ENOUGH TO BE SIGNIFICANT AT THE 1 PER CENT AND THE 5 PER CENT LEVELS OF CONFIDENCE

	TWENTY MEN (190 PAIRINGS)			FORTY WOMEN (780 PAIRINGS)		
	RESTING PULSE	ONE-HALF MINUTE AFTER EXERCISE	INCREASE DUE TO EXERCISE	RESTING PULSE	ONE-HALF MINUTE AFTER EXERCISE	INCREASE DUE TO EXERCISE
Population variance based on individual variability	41.1	45.2	34.4	32.6	47.7	49.2
Standard error M_1-M_2	4.53	4.75	4.15	4.05	4.88	4.96
Difference required at 1 per cent level	17	18	15	15	18	18
Difference required at 5 per cent level	11	11	10	10	12	12
Number of differences meeting 1 per cent requirement	15	77	73	197	251	223
Number of differences meeting 5 per cent requirement	52	117	112	356	413	391
Per cent of differences meeting 1 per cent requirement	8	41	38	25	32	29
Per cent of differences meeting 5 per cent requirement	27	62	59	46	53	50

Since some individuals are more variable than the average with respect to pulse scores, some of the differences as large as those specified in Table III will not yield a significant t-ratio (it equals the difference between two means divided by the standard error of that difference). Conversely when the standard error of the difference is small, differences less than the stated number of beats will be significant. These discrepancies balance each other however, so that the actual number of significant differences is approximately the same as the number of differences predicted in Table III. This was checked em-

pirically after computing a series of *t*-tests for the significance of the differences in pulse one-half minute after exercise among the twenty college men.

This analysis shows that the resting pulse has some discriminatory value, yielding more significant differences than would be expected by chance. It also shows the extent to which the pulse after exercise is superior to the resting pulse. And finally it calls attention to the limitations of even the best of the cardiovascular tests used in this experiment; the average of four pulse scores one-half minute after a standard exercise was able to distinguish only 41 per cent of the time at the 1 per cent level of confidence and 62 per cent of the time at the 5 per cent level.

Reliability Coefficients.—The discriminatory power of a test can also be inferred from the coefficients obtained when two sets of scores on the same subjects are correlated. Such coefficients become larger with an increase in the range of scores around the group mean, and as the variability with respect to individual means decreases.

The reliability coefficients obtained from our data on the twenty college men and forty college women are given in Table IV. The coefficients under the heading I II were obtained by correlating the first set of scores with the second. The reliability of the average of four determinations ('44) was estimated from 'I II) (III IV) by means of the Spearman-Brown formula.⁴

If the degree of reliability represented by a coefficient of .85 or above is desired in cardiovascular testing, it is necessary to use the average of four determinations rather than single test scores.

TABLE IV
RELIABILITY COEFFICIENTS FOR SINGLE DETERMINATIONS OF HEART RATE AND BLOOD PRESSURE
AND FOR THE AVERAGE OF FOUR DETERMINATIONS

	RESTING		ONE-HALF MINUTE AFTER EXERCISE	
	'I II	'44	'I II	'44
Heart Rate				
Men	.63	.83	.86	.94
Women	.76	.86	.78	.90
Systolic Pressure				
Men	.74	.87	.60	.78
Women	.88	.93	.74	.88
Diastolic Pressure				
Men	.71	.86	.71	.87
Women	.72	.86	.76	.92

CONCLUSIONS

1. Pulse after a standard exercise is a more reliable measure than the resting pulse, but the resting systolic blood pressure is a more reliable measure than the systolic pressure after exercise.
2. Even when conditions are carefully controlled, an individual's heart rate and blood pressure are so variable from day to day that a number of determinations must be made if his general status is to be established.
3. Cardiovascular tests in general have little discriminatory power because the differences in scores among individuals are small in relation to individual variability.

4. Individuals can more clearly be distinguished from each other on the basis of postexercise pulse rates than on the basis of resting heart rate or the increase due to moderate exercise. The same is true of postexercise systolic and diastolic blood pressures.

5. The relative efficiency of heart rate scores in distinguishing individuals from each other has been demonstrated in terms of the percentage of significant differences among individuals in a group. Whereas only 8 per cent of the differences in the resting pulse for the twenty men in our experiment are significant at the 1 per cent level of confidence, 41 per cent of the differences in pulse one-half minute after exercise are significant at this level. The corresponding figures for the women are 25 per cent and 32 per cent. At the 5 per cent level of confidence, 62 per cent of the differences in pulse after exercise are significant among the men; among the women only 53 per cent of the differences are significant at this level of confidence.

REFERENCES

1. Nicholson, Everett: A Study of Fluctuation in Physical Efficiency, M. A. thesis, 1942, State University of Iowa Library.
2. Lindquist, E. F.: Statistical Analysis in Educational Research, New York, 1940, pp. 60-66, Houghton Mifflin Co.
3. Lindquist, E. F.: Statistical Analysis in Educational Research, New York, 1940, pp. 51-59, Houghton Mifflin Co.
4. Garrett, H. E.: Statistics in Psychology and Education, New York, 1937, p. 315, Longmans, Green & Co.

THE OSCILLOMETER AND THERMOCOUPLE AS DIAGNOSTIC AIDS IN PERIPHERAL VASCULAR DISEASE

CAMPBELL MOSIS, M.D., AND MURRAY B. FERDERBER, M.D.
PITTSBURGH, PA.

THE widespread use of the oscillogram and thermocouple as diagnostic aids in peripheral vascular disease occasioned this study. An attempt was made to record the evidence obtained by various diagnostic procedures and to correlate this evidence with the circulation in the extremities examined.

TECHNIQUE

To maintain standard conditions for all the observations, an air-conditioned room was equipped in the Falk Clinic. In this room it was possible to maintain both the temperature and the humidity within a narrow range. A temperature of 75° F. and a relative humidity of 75 per cent were used as standard, and all observations were made under these conditions.

Data relative to the peripheral circulation were obtained on 102 subjects. Of these, 48 were normal individuals, 8 were asymptomatic with relation to peripheral vascular disease although they had some other systemic disease, 6 had thromboangiitis obliterans, and 28 had arteriosclerotic vascular disease. Of the 12 remaining patients, varicosities, neuritis, and angina pectoris accounted for all but 1. This patient was a Negress in whom no arterial pulse could be obtained in the upper extremities.

The sex, age, and race and the location, character, and duration of the symptoms of each patient were noted (Fig. 1). Previous treatment received by the patient and measures that afforded relief from symptoms as well as the family and past medical history were recorded. Physical examination, including a neurological examination, was given each patient.

Alterations in the color of the lower extremity in the dependent, horizontal, and elevated positions were noted. A period of ten minutes in each of these positions was allowed before the color change was recorded. Abnormal pigmentation, the presence or absence of phlebitis, ulcers, gangrene, edema, trophic changes in the skin, nails, and hair, and pulsations of the dorsalis pedis and posterior tibial arteries were observed. Calf measurements were recorded at the level of the greatest circumference.

Skin temperature observations were obtained with a skin temperature thermocouple apparatus in the following manner. With the subject in the sitting position, the lower extremities were exposed in the air-conditioned room

From the Department of Industrial Hygiene of the University of Pittsburgh School of Medicine

The Westinghouse Electric Supply Company equipped the air-conditioned room at the Falk Clinic on the recommendation of Dr. T. Lyle Hazlett, Medical Director of Westinghouse Electric & Manufacturing Co.

The subjects used in this study were largely taken from the diabetic and medical services of the Falk Clinic and from the student body of the University of Pittsburgh. Additional patients were seen through the courtesy of Dr. T. Lyle Hazlett, Dr. Joseph H. Barach, Dr. W. Leigh Cook, Jr., Dr. W. M. McClements, and Dr. L. L. Pennoek.

Received for publication, May 12, 1944.

for a period of twenty-five minutes. Skin temperatures were then recorded from the anterior portion of the thigh, the patella, the mid-calf, the internal and external malleoli, the dorsum of the foot, and the mid-plantar surface and from the skin at the base of the great toenail. The legs were then placed in the horizontal position upon a low, padded stool. After they had remained in this position for ten minutes, skin temperatures were again recorded from the areas noted above. Finally the patient was placed in the supine position upon an examining table with the lower extremities supported at an angle of 45 degrees above the horizontal. Skin temperatures were again recorded after a similar stabilizing period. The skin temperature findings from the thigh to the great toe for each subject in each of the three positions were plotted to illustrate the centrifugal variation in skin temperature.

A recording oscillogram was used to obtain the oscillographic readings. The cuff was placed about the calf and inflated to a level somewhat above the systolic blood pressure. The pressure in the system was then decreased by 10 to 20 mm. Hg in successive steps until no pulsation was noted.

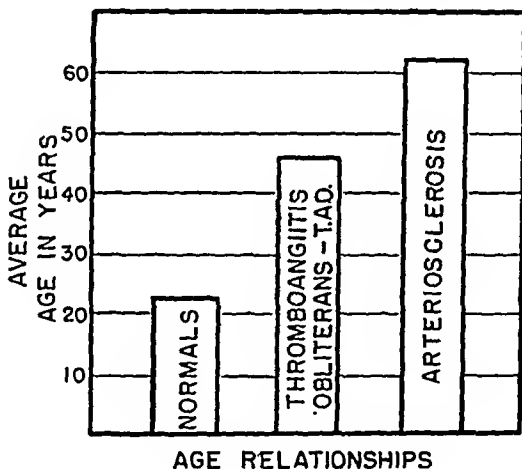


Fig. 1.

Vibratory sensation was tested according to the method of Barach.¹ An especially constructed tuning fork was used which made it possible to observe the instant when a standard amplitude of vibration was obtained. The length of time which elapsed from the instant the standard amplitude of vibration was reached until the subjective sensation disappeared was recorded with a stop watch. Vibratory sensation determinations were made at the angle of the sternum, the lower end of the radius and ulna, the anterior superior spine of the ilium, the mid-point of the tibia, the internal and external malleoli, and over the tip of the sacrum. These readings were then individually plotted against the normal variation previously obtained by Barach.

At the suggestion of Dr. C. C. Guthrie, the calorimetric method of estimating the blood flow in the extremities devised by Stewart¹⁴ was used. Through the courtesy of Dr. J. M. Rogoff, the original calorimeters which Stewart devised were available, and with these as models, suitable calorimeters were constructed. The technique described by Stewart was rigidly followed.

This technique is summarized¹¹ briefly. The subject to be examined placed the extremities in a bath containing water at approximately 30° C. At the end of fifteen minutes the part to be tested was transferred swiftly and with a minimum of exposure into a calorimeter containing water at exactly the same temperature as that in the preliminary bath. The water within the calorimeter was stirred by means of feathers, and temperature readings estimated to the hundredth of a degree were taken at regular intervals for twenty minutes.

From the transfer of heat from the part being tested to the water bath it was possible to estimate the flow of blood through the part by the following formula:

$$Q = \frac{H}{T - T^1} \times \frac{1}{S}$$

Where Q is equivalent to the quantity of blood flowing through the part during the period of observation; H is the heat given off to the calorimeter during the period of observation and the water equivalent of the calorimeter and of the part. T represents the temperature of the arterial blood. Stewart's figure for this is 0.5° C. below the rectal temperature or the equivalent of oral temperature. T^1 equals the temperature of the venous blood. Because of the heat transfer that takes place between the water in the calorimeter and the superficial veins, the temperature of the venous blood was taken to be equal to the mean temperature of the calorimeter. S represents the specific heat of blood, or 0.9.

The volume of the part through which the blood flow was being determined was measured by the displacement of water. The flow of blood was recorded in grams of blood per 100 c.c. part volume per minute. As pointed out by Sheard,¹² the actual determination of the volume flow of blood by this method is open to criticism.

OBSERVATIONS

Of the 48 normal subjects in the group examined, 39 were men and 9 women. The average age of the normal group was 23 years. All 6 with thromboangiitis obliterans were males; they had an average age of 45.5 years; 3 members of this group were Hebrew. The average age of the 28 individuals with arteriosclerosis was 61.7 years; of this group 14 were men and 14 women (Fig. 1).

In the arteriosclerotic group 23 of the 28 patients were diabetic. A history of neuritis was obtained in one-half of the patients who were diabetic. Either history or evidence of an active phlebitis was present in 3 of the 6 patients with thromboangiitis obliterans. Only 1 of the 28 subjects with arteriosclerosis gave a history of phlebitis. None in the normal or asymptomatic group had had phlebitis.

In the subjects with arteriosclerosis of the lower extremities, intermittent claudication was a common symptom; almost as frequently the pain was noted in the ankle, dorsum, longitudinal arch, or in the toes. Numbness, tingling, burning, aching, and rest pain were common symptoms in those in this group. A sense of local fatigue rather than intermittent claudication was often an early sign of peripheral vascular involvement. Of the patients with arterio-

sclerotic vascular disease who exhibited rest pain, almost 50 per cent were able to obtain relief from the pain by moderate exercise, warmth, or massage. None of the patients with thromboangiitis obliterans found the rest pain relieved by exercise. Both the patients in the arteriosclerotic group and those with Buerger's disease obtained relief from intermittent claudication by rest.

A variable decrease from the normal calf measurement was noted in subjects with peripheral vascular disease (Fig. 2). Atrophic changes in the skin were noted in over 50 per cent of those with arteriosclerosis. Only 25 per cent of this group had a normal distribution of hair over the extremities. As would be expected from the variation in the age groups, the subjects with arteriosclerosis had a higher average blood pressure than did those with Buerger's disease.

Palpable varicosities were noted in over 75 per cent of the subjects with symptoms of arteriosclerotic vascular disease. One-half of this group consisted of older women, many of whom had had large families; this probably accounts for the high incidence of varicosities. However, it is not unlikely that the symptoms of vascular disease are more prone to appear in the presence of both varicosities and arteriosclerosis.

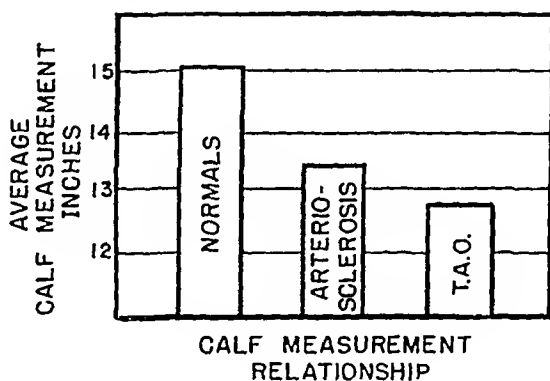


Fig. 2.

Merely the presence of rubor, cyanosis, or pallor is not of itself indicative of vascular disease. Included in this series were both normal and symptomatic subjects with these signs. Rubor was commonly seen in normal individuals with the feet in a dependent position when the room temperature exceeded 78° F. Edema of the lower extremities of some degree was found in 45 per cent of those in the arteriosclerotic group. However, in interpreting this figure it must be recalled that one-half of those in this group also had palpable varicosities. Three of the patients with thromboangiitis had demonstrable edema of the legs.

The pulsation of the dorsalis pedis artery was palpable in all but 2 of the normal individuals; significantly it was also palpable in over one-half of those with arteriosclerotic vascular disease. This pulsation was present most frequently in those with mild evidence of vascular disease. The posterior tibial pulsation was absent frequently in the normal subjects, but was palpable in over 20 per cent of those with arteriosclerosis. It was noted that when the posterior tibial pulsation was not palpable, it could frequently be visualized by everting the foot. This evidence supports the well-known clinical conclusion

that the absence of the dorsalis pedis or posterior tibial pulsation does not necessarily indicate the presence of vascular disease; and, conversely, the presence of a palpable pulsation does not rule out the possibility of arterial vascular disease.

The oscillometric findings did not indicate unequivocally the presence or degree of involvement of peripheral vascular disease. The average reading obtained in patients with symptoms of arteriosclerotic vascular disease were slightly higher than those obtained in normal individuals (Fig. 3). This may be explained by the fact that in those with chronic arterial disease the musculature of the leg is weak and somewhat atrophic. The artery may then be somewhat superficially situated and each impulse of the artery is transmitted more readily to the inflated cuff than it is in the individual with rigid, heavy musculature. In the presence of arteriosclerosis, if the hardened artery is superficially located, it may rise out of its bed with each thrust of the pulse; this motion may be transmitted to the oscillometer and recorded as a normal arterial pulsation when actually the blood flow to the part is inadequate due to the diminished lumen of the vessel. Conversely, diminished oscillometric

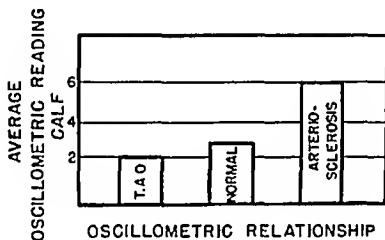


Fig. 3.

readings may be obtained in normal individuals with heavy musculature because the arterial impulse is poorly transmitted as the artery lies in a bed of loose areolar tissue with firm muscular support.

While diminished oscillometric readings could not be interpreted as being absolutely indicative of vascular disease, it was possible with repeated oscillometric studies in the same individual to determine from time to time the changes in peripheral pulsation that occurred. The evidence obtained by repeated oscillometric observations on the course of a vascular disease, is, in our opinion, one of the chief uses of the oscillometer today.

The skin temperatures of all subjects in the dependent, horizontal, and elevated positions were recorded and no significant or constant changes were noted in any of these positions. No significant changes in skin temperature occurred in normal, arteriosclerotic, or thromboangiitic extremities with the legs in the dependent, horizontal, or elevated positions.

Of 384 observations on the leg in the dependent position, 424 in the horizontal, and 368 in the elevated position, it was found that the average skin temperature in each position was very slightly higher in the arteriosclerotic extremity than in the normal extremity.

Chronic vascular disease of the larger arteries does not necessarily result in decreased skin temperatures. The temperature of the skin is obviously dependent upon many factors, one of these being arteriovenous anastomoses which shunt blood directly from the arterioles into the superficial veins.⁶ By this means the blood may be directed into the cutaneous tissues before it has been cooled by passing through the capillary circulation. Since certain types of arteriosclerosis present pathologic lesions of the larger arteries but not, to any great extent, of the arterioles, it is entirely possible by means of this arteriovenous shunt to have a normal skin temperature although the circulation of the part may be impaired. This mechanism is only one factor that renders the interpretation of skin temperatures difficult; the temperature of the examining room, the previous activity of the part, the clothing worn by the patient, the psychic state of the patient, and many other factors may cause reflex changes in the circulation of an extremity and thereby alter the skin temperature.

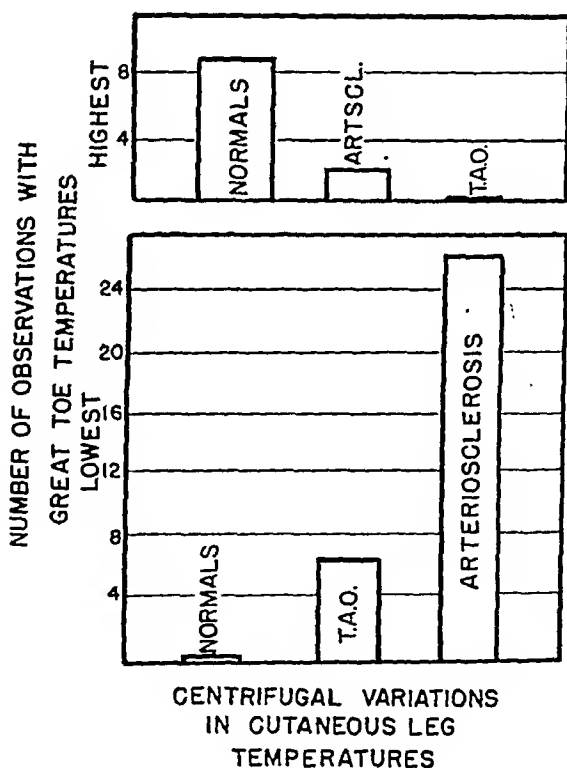


Fig. 4.

Possibly a more satisfactory apparatus than the thermocouple for determining skin temperature is the radiometer; this device has the advantage of measuring the radiated heat from a larger surface area. With the thermocouple device it is possible to unknowingly place the metallic contact close to a superficial vessel and thereby obtain an excessively high reading. It is our opinion that a reliable interpretation of a patient's skin temperature may be made by the examiner placing his hand briefly on the extremity in various locations.

Obviously the hand should be warmed to room temperature. The impression of the skin temperature that is obtained by palpation gives, in our opinion, a more accurate index of the circulatory status of the extremity than is obtained by the use of the thermocouple. From the data presented here it is apparent that normal skin temperatures as obtained with the thermocouple do not exclude arterial vascular disease.

It is generally recognized that there is a centrifugal decrease in skin temperature. It is interesting to note that in this study the temperature of the great toe was the highest recorded on the entire leg in 16 observations; in 40 individuals the great toe temperature was the lowest obtained; and in 46 the temperature of the great toe was somewhere between the highest and lowest recorded on that extremity (Fig. 4). The dorsum of the foot was the warmest part in 36 patients, 26 of these having arteriosclerotic vascular disease. Again the explanation of these alterations in skin temperature may lie in the altered cutaneous blood supply caused by changes in the arteriovenous anastomoses. The centrifugal decrease in the temperature of the arterial blood is not disputed, but since the arteriovenous anastomoses have been found mainly in the distal portion of the extremities, the increased cutaneous circulation may permit the skin to have a higher temperature peripherally than centrally.

Studies on the blood flow in the feet by Stewart's technique indicated that the blood flow in patients with arteriosclerotic vascular disease and thromboangiitis obliterans averages about two-thirds that obtained in normal subjects (Fig. 5). It is to be noted, however, that a wide range of values may be

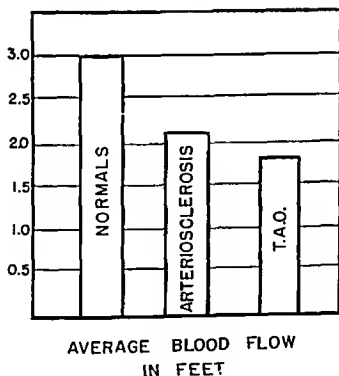


Fig 5

regarded as normal. Slight errors in technique or alterations in the environment markedly alter the results. To obtain comparable readings in a series of patients it is essential that they be examined under exactly the same conditions.

Sheard¹² has criticized this method of quantitatively estimating the circulation. For this reason no quantitative figures of blood flow are offered. Comparative blood flow readings that are very high or very low when obtained

under standard conditions may be interpreted readily, but readings in the wide borderline range of normal values cannot be used as an accurate index of the circulation of the part. Determination of the blood flow by this method is of little use clinically, and other probably more satisfactory methods are available for investigative purposes.

Estimation of vibratory sensation by the method described yields confirmatory evidence of deficient circulation. Of the individuals examined in this study, all those with obviously deficient circulation had diminished vibratory sensation. However, diminution in vibratory sense is a late sign of circulatory deficiency and is of little diagnostic value in early cases. It is probably a confirmatory sign of the nervous deterioration accompanying impaired circulation.

SUMMARY AND CONCLUSIONS

1. Observations relative to the peripheral circulation were made on 102 individuals. This group included 48 normal subjects, 8 asymptomatic, 6 with thromboangiitis obliterans, and 28 with arteriosclerosis. Observations as to history, symptoms, physical signs, oscillometric readings, skin temperatures, blood flow, and vibratory sensation were noted and the results tabulated.

2. The techniques used in obtaining the data are briefly described.

3. While in arteriosclerosis of the lower extremity, symptoms referable to the calf were most common, pain was noted almost as frequently in the ankle, arch, dorsum, or toes. Numbness, tingling, burning, aching, and rest pain were symptoms noted almost as frequently as intermittent claudication in arteriosclerotic vascular disease. A feeling of local fatigue was often the first sign of vascular disease.

4. Arteriosclerotic rest pain was often relieved by moderate exercise. The pain of thromboangiitis obliterans was not relieved by exercise in any of our patients.

5. Normal distribution of the hair over the lower extremity was noted in only 25 per cent of the patients with vascular disease.

6. Rubor, cyanosis, or pallor of an extremity may be present in the absence of arterial vascular disease.

7. The absence of the *dorsalis pedis* or posterior tibial pulsation was not pathognomonic of vascular disease, and the presence of a pulsation did not exclude vascular pathology.

8. The oscillometric readings were not necessarily found to be decreased in arteriosclerotic peripheral vascular disease; normal individuals with heavy musculature may have diminished oscillometric readings.

9. Normal skin temperatures may obtain in individuals with vascular disease.

10. Estimation of the blood flow by Stewart's calorimetric method indicated that the blood flow in patients with vascular disease averaged about two-thirds of that in normal subjects.

11. Estimation of the vibratory sense by the method of Barach yields confirmatory evidence of deficient circulation but is of little early diagnostic value.

12. The arteriovenous anastomoses are suggested as one mechanism partially explaining the variations in skin temperature that occur in normal individuals and those with peripheral vascular disease.

REFERENCES

1. Barach, J. H.: Unpublished observations.
2. Clark, E. R.: Arterio-Venous Anastomoses, *Physiol. Rev.* 18: 229, 1938.
3. Grant, R. T., and Bland, E. F.: Observations on Arterio-Venous Anastomoses in Human Skin and in the Bird's Foot With Special Reference to the Reaction to Cold, *Heart* 15: 256, 281, 385, 1930; 16: 69, 1932.
4. Grant, R. T.: Observations on Blood Circulation in Voluntary Muscle in Man, *Clin. Sc.* 3: 157, 1938.
5. Harpuder, Karl, Stein, Irvin, and Byer, Jacob: Effect of Arterio-Venous Shunt in Peripheral Vascular Disease, *Arch. Phys. Therapy* 19: 272, 1938.
6. Harpuder, Karl, Stein, Irvin, and Byer, Jacob: The Role of the Arterio Venous Anastomosis in Peripheral Vascular Disease, *Am. Heart J.* 20: 5, 1940.
7. Krogh, A.: The Anatomy and Physiology of the Capillaries, New Haven, 1922, Yale University Press.
8. Leriche, R., and Weigun: Effects of Arterial Ligature on Vasomotor System, *Lancet* 2: 296, 1940.
9. Lewis, T.: Pathological Changes in Arteries Supplying Fingers in Warm Handed People and in Cases of So-called Raynaud's Disease, *Clin. Sc.* 3: 287, 1938.
10. Pollicard, A., editor: *Histophysiologie IV: Les glanues neuro-vasculaires*, par Pierre Masson, Paris, 1937, J. Hermann.
11. Popoff, N. W.: The Digital Vascular System, *Arch. Path.* 18: 295, 1934.
12. Sheard, Charles, Keggereis, Roy, and Brown, George E.: Calorimetric Studies of the Extremities, *J. Clin. Investigation* 3: 327, 1926.
13. Steia, I. D.: Studies of Collateral Circulation Following Experimental Vascular Occlusion, *Am. Heart J.* 14: 726, 1937.
14. Stewart, G. N.: Studies on the Circulation—Harvey Lectures, Baltimore, Md., 1912-13, Williams & Wilkins Co.
15. Suequet, J. P.: D'une Circulation Derivative Dans Les Membranes et Dans la Tete Chez L'homme, Paris, 1862.
16. Wright, I. S., and Duryee, A. W.: Human Capillaries in Health and Disease, *Arch. Int. Med.* 52: 545, 1933.

A STUDY ON THE RELATIONSHIP OF BLOOD ISO-AGGLUTININ TITERS TO TOTAL SERUM PROTEIN CONCENTRATION

ALBERT P. ROWE, JR., M.D., ALICE MCBRIDE, A.B., AND STACY R. METTIER, M.D.
SAN FRANCISCO, CALIF.

THE role of blood iso-agglutinins in whole blood and plasma transfusion reactions now is established. With the current renewal of interest in protein metabolism and the widespread use of plasma and protein hydrolysates in combating hypoproteinemia, the behavior of blood iso-agglutinin titers, with changes in serum protein concentration, assumes importance from a clinical and experimental standpoint. The present study demonstrates that no definite relationship exists between blood iso-agglutinin titers and total serum protein concentrations.

There are certain well-established facts regarding the behavior of blood iso-agglutinins in man. Minor variations in serum titers occur from time to time and without apparent cause in normal persons. The titer is reduced in middle-aged and elderly persons. Increased titers have been observed during acute infections, in heterospecific pregnancies,⁵ and after plasma transfusions.⁶ Davidsohn⁷ reported very high titers in patients with sickness resulting from the administrations of horse serum and low titers in patients with severe anemia or chronic leucemia.

METHODS

Samples of blood for total serum protein determinations and titration of iso-agglutinins were collected according to the standards of Kagan,¹ which require that the blood be drawn within one minute after application of the tourniquet; the samples must be kept and centrifuged in well-stoppered tubes; only non-hemolyzed serum can be used, and no amount of fluid greater than 500 c.c. should be ingested at any time within four hours before taking the sample.

Total serum protein determinations were made with the Kagan proteinometer* by the falling drop method.^{2, 3} A few determinations were carried out in the hospital clinical laboratory by the colorimetric method. The normal range of total serum protein concentration was considered to be from 6.0 to 7.5 Gm. per 100 c.c., as determined by Kagan.¹

Titration of serum iso-agglutinins was performed by making successive dilutions of serum in 7 mm. test tubes with physiologic saline solution. To each test tube was added 3 drops of a 1.0 per cent suspension of A or B cells in physiologic saline. Fresh cell suspensions were prepared daily from two healthy adults. The cell-serum mixtures were shaken vigorously and incubated in a water bath at 37.5 C. for thirty minutes. Each tube then was centrifuged

From the Division of Medicine, University of California Medical School.

Supported by a grant from the Council on Pharmacy and Chemistry of the American Medical Association.

Received for publication, June 27, 1944.

*The Kagan proteinometer is manufactured by E. H. Sargent & Co., Chicago, Ill.

at 500 r.p.m. for one minute, after which the cells were resuspended by a gentle but thorough shaking. Titration end points were determined by low-power microscopic examination of the suspension. The highest dilution which contained clumps of three or more cells was regarded as the end point. Only differences of two or more dilutions were considered significant in individual patients who were observed at intervals.

Amigen* powder was administered by dissolving the preparation in hot broth. Dosage varied from 6 to 8 tablespoonsfuls (from 54 to 72 Gm.) in approximately 200 c.c. of broth two to three times daily.

All technical procedures, except the few colorimetric protein determinations, were carried out by us.

CLINICAL MATERIAL

Normal persons were selected at random for these studies from the medical and nursing staffs and the medical classes of the University of California Hospital. All had serum protein concentrations within the expected normal range. With one exception, all the patients with hypoproteinemia were in the same hospital.

Three patients with hypoproteinemia received amigen orally to replenish depleted body protein stores and raise circulating serum protein levels. Two of these patients received whole blood transfusions repeatedly. In no instance were titrations performed earlier than three days after transfusion. Although no significant changes in titer have been observed subsequent to compatible blood transfusions, elevations have been noted after the injection of plasma. Wiener⁷ suggests that the presence of red cell stroma may contribute to the immunization effect.

A. W., a 29-year-old white man, noted extreme weakness, pallor, and dyspnea six months prior to his entry to the hospital April 5, 1943. A diagnosis of aplastic anemia was established by examination of the peripheral blood and bone marrow. The initial blood count revealed the hemoglobin to be 1.5 Gm. (8 per cent) and the red blood cells to be 440,000 per cubic millimeter. The presence of severe hypoproteinemia was discovered by the finding of a total serum protein concentration of 4.27 Gm. per cent. The treatment during the initial hospital stay of one month consisted of whole blood transfusions, liver extract administered parenterally, ferrous sulfate, vitamin C, and amigen (from 54 to 72 Gm. twice daily) by mouth. During the initial period the patient developed purpura on one occasion and a number of large furuncles. The furuncles cleared rapidly. Upon discharge from the hospital the hemoglobin was noted to be 80 per cent. There were six subsequent hospital entries for transfusion of whole blood or red cell mass. The treatment that he received while ambulatory was similar to that which had been instituted in the hospital. Death occurred Oct. 11, 1943, from a cerebral hemorrhage during hospitalization for a recurrence of purpura. Autopsy confirmed the diagnosis of aplastic anemia.

H. W., a 46-year-old Jewish man, noted weakness for one month before entering the hospital March 18, 1943. Two weeks prior to admission he had been disturbed by dyspnea on exertion, tinnitus, and precordial pain. A blood count taken March 17, 1943, because of suspected lymphangitis of one arm revealed: hemoglobin, 4.6 Gm. (32 per cent); red blood cells, 1.64 million; and white blood cells, 2,250. Additional blood and bone marrow studies resulted in a diagnosis of aplastic anemia. The treatment for this patient was identical to that employed in the patient in preceding case, but purpura appeared early and

*Amigen is a dried enzymic digest of purified casein and pork pancreas containing amino acids and polypeptides kindly furnished by Mead Johnson & Co., Evansville, Ind.

rectal and gingival bleeding became severe. There was progressive reduction of the hemoglobin concentrations. The patient was discharged from the hospital June 30, 1943; the hemoglobin concentration was 94 per cent, and there was severe bleeding from the gums and extensive retinal hemorrhages. In spite of transfusions, bleeding increased and death occurred at home July 31, 1943. An autopsy was not performed.

H. S., a 48-year-old white man, was proved to be suffering from chronic glomerulonephritis in the nephrotic stage. Treatment consisted of a low salt, high protein diet which was supplemented with amigen in 54 to 72 Gm. doses twice daily. There was little change in the total serum protein concentration over a five-week period. The patient was discharged from the hospital May 20, 1943, and failed to return for follow-up studies.

OBSERVATIONS

The twenty normal subjects reported in Table I had the usual distribution of blood types and a range of total protein concentrations within normal limits. In this group there was no constant relationship between iso-agglutinin titers

TABLE I
NORMAL SUBJECTS

SUBJECT	TYPE	TOTAL SERUM PROTEIN (GM.)	ANTI-A TITER	ANTI-B TITER
1 (M. D.)	B	6.18	1:4096	
2 (M. A.)	O	6.28	1:1024	1:512
3 (A. B.)	A	6.30		1:1024
4 (D. G.)	O	6.39	1:4096	1:1024
5 (H. K.)	A	6.42		1:512
6 (S. M.)	O	6.46	1:8192	1:8192
7 (M. M.)	A	6.49		1:2048
8 (M. L.)	A	6.53		1:16384
9 (F. L.)	O	6.62	1:4096	1:2048
10 (B. G.)	O	6.62	1:4096	1:512
11 (J. R.)	O	6.62	1:8192	
12 (D. R.)	B	6.77	1:2048	
13 (M. S.)	O	6.90	1:2048	1:512
14 (N. C.)	O	6.97	1:65536	1:16384
15 (M. B.)	A	7.08		1:16384
16 (M. W.)	A	7.22		1:2048
17 (A. W.)	A	6.10		1:512
18 (M. S.)	O	7.03	1:4096	1:2048
19 (C. H.)	A	6.76		1:2048
20 (S. D.)	A	6.87		1:512

TABLE II
HYPOPROTEINEMIC SUBJECTS
SINGLE OBSERVATIONS

SUBJECT	TYPE	TOTAL SERUM PROTEIN (GM.)	ANTI-A TITER	ANTI-B TITER
1 (A. C.)	B	4.74	1:512	
2 (A. B.)	A	4.97		1:1024
3 (L. S.)	B	5.56	1:256	
4 (O. U.)	O	5.59	1:256	1:512
5 (D. D.)	O	5.69	1:8192	1:1024
6 (P. C.)	A	5.71		1:16384
7 (B. C.)	A	5.72		1:2048
8 (F. S.)	A	5.82		1:1024
9 (B. L.)	O	5.82	1:2048	1:512

and serum protein levels. As has been noted by other investigators, anti-A titers were higher than anti-B titers. The high anti-A titer in one subject 14 (N. C.), was checked repeatedly but remains unexplained.

Single determinations of iso-agglutinin titers in patients with hypoproteinemia are recorded in Table II. Hypoproteinemia was mild in the majority of patients and the titers of the entire group evinced no significant difference when compared with the normal subjects. No correlation existed between the degree of hypoproteinemia and iso-agglutinin concentrations.

TABLE III
PATIENT A. W.
TYPE O

DATE (1943)	TOTAL SERUM PROTEIN (Gm.)	ANTI-A TITER	ANTI-B TITER
3/24	5.37	1:1024	1:512
3/25	5.37	1:1024	1:512
4/7	4.26		
4/12	5.66	1:4096	1:2048
4/20	7.11	1:4096	1:1024
4/27	6.49	1:4096	1:1024
5/5	5.66	1:8192	1:2048
5/10	6.14	1:8192	1:4096
5/18	6.18	1:2048	1:4096
6/3	5.70	1:16384	1:4096
6/18	6.25	1:16384	1:16384
6/25	5.97	1:16384	1:16384
6/28	6.35	1:16384	1:8192
7/9	6.49	1:8192	1:8192
7/27	6.11	1:16384	1:16384
8/14	6.42	1:8192	1:8192
8/30	6.14	1:8192	1:8192
9/10	6.07	1:16384	1:16384
9/22	5.80	1:16384	1:16384
9/30	5.70	1:16384	1:16384
10/8	5.45	1:16384	1:16384

The iso-agglutinin titers in one patient (A. W. Table III) rose from the initial concentrations of anti-A 1:1,024 and anti-B 1:512 to concentrations of anti-A 1:8,192 to 1:16,384 and anti-B 1:8,192 to 1:16,384. Although there was no constant relationship between single serum protein concentrations and iso-agglutinin titers, nevertheless there appeared to be a fairly well maintained rise in iso-agglutinin titers when the serum protein concentration was maintained at levels above 5.70 Gm. per cent. In the case of patient H. W. (Table IV) there was no correlation between the serum protein concentration and iso-agglutinin titers; in fact, the lowest titers (1:16 and 1:32) were found with normal serum protein levels. Patient H. S. (Table V) had serum protein concentrations which were consistently low and which varied between 3.93 and 4.11 Gm. per cent. Anti-A and anti-B titers showed variations of only three dilutions—1:128 to 1:1,024. Minor elevations in serum protein concentrations had no constant effect on iso-agglutinin titers.

rectal and gingival bleeding became severe. There was progressive reduction of the hemoglobin concentrations. The patient was discharged from the hospital June 30, 1943; the hemoglobin concentration was 94 per cent, and there was severe bleeding from the gums and extensive retinal hemorrhages. In spite of transfusions, bleeding increased and death occurred at home July 31, 1943. An autopsy was not performed.

H. S., a 48-year-old white man, was proved to be suffering from chronic glomerulonephritis in the nephrotic stage. Treatment consisted of a low salt, high protein diet which was supplemented with amigen in 54 to 72 Gm. doses twice daily. There was little change in the total serum protein concentration over a five-week period. The patient was discharged from the hospital May 20, 1943, and failed to return for follow-up studies.

OBSERVATIONS

The twenty normal subjects reported in Table I had the usual distribution of blood types and a range of total protein concentrations within normal limits. In this group there was no constant relationship between iso-agglutinin titers

TABLE I
NORMAL SUBJECTS

SUBJECT	TYPE	TOTAL SERUM PROTEIN (GM.)	ANTI-A TITER	ANTI-B TITER
1 (M. D.)	B	6.18	1:4096	
2 (M. A.)	O	6.28	1:1024	1:512
3 (A. B.)	A	6.30		1:1024
4 (D. G.)	O	6.39	1:4096	1:1024
5 (H. K.)	A	6.42		1:512
6 (S. M.)	O	6.46	1:8192	1:8192
7 (M. M.)	A	6.49		1:2048
8 (M. L.)	A	6.53		1:16384
9 (F. L.)	O	6.62	1:4096	1:2048
10 (B. G.)	O	6.62	1:4096	1:512
11 (J. R.)	O	6.62	1:8192	
12 (D. R.)	B	6.77	1:2048	
13 (M. S.)	O	6.90	1:2048	1:512
14 (N. C.)	O	6.97	1:65536	1:16384
15 (M. B.)	A	7.08		1:16384
16 (M. W.)	A	7.22		1:2048
17 (A. W.)	A	6.10		1:512
18 (M. S.)	O	7.03	1:4096	1:2048
19 (C. H.)	A	6.76		1:2048
20 (S. D.)	A	6.87		1:512

TABLE II
HYPOPROTEINEMIC SUBJECTS
SINGLE OBSERVATIONS

SUBJECT	TYPE	TOTAL SERUM PROTEIN (GM.)	ANTI-A TITER	ANTI-B TITER
1 (A. C.)	B	4.74	1:512	
2 (A. B.)	A	4.97		1:1024
3 (L. S.)	B	5.56	1:256	
4 (O. U.)	O	5.59	1:256	1:512
5 (D. D.)	O	5.69	1:8192	1:1024
6 (P. C.)	A	5.71		1:16384
7 (B. C.)	A	5.72		1:2048
8 (F. S.)	A	5.82		1:1024
9 (B. L.)	O	5.82	1:2048	1:512

A STUDY OF STAPHYLOCOCCUS ANTITOXIN TITERS IN NORMAL HUMAN SERA

DOROTHY DIXON SEELY, M.A., AND PAUL F. STOOKEY, M.D., KANSAS CITY, Mo.,
AND NOBLE P. SHERWOOD, M.D., LAURENCE, KAN.

WITH the frequent therapeutic use of specific antitoxin in acute staphylococcus infections and of toxoid in those cases of chronic manifestations, the laboratory is being called upon more and more for determinations of the quantity of staphylococcus antitoxin in the patient's blood serum. To interpret laboratory examinations of any kind, it is necessary to know the results of the test in normal individuals and to evaluate the pathologic sample accordingly. Although an approximate normal value for circulating staphylococcus antitoxin has been known for some time, we felt that it would be expedient and worth while to test a large group of sera to establish an average normal and also a normal titer range. The purpose of this paper is to present the background, the technique, and the results of this problem.

Early reports of staphylococcal antihemolysin content are not readily comparable because of differences of standards or units employed in the various laboratories. The earliest reports in this country of titrations done to estimate staphylococcus antitoxin are those of Bryee and Burnet in 1932.¹ They did a series of normal titers on mothers and newborn infants, reporting a wide variation in titer; different individuals showing as little as 2 units or as much as 650 units, with the majority showing 40 to 160 units. The technique did not involve the use of international units and simply used an arbitrary "unit" value. The authors admitted a technical error in the titer readings of minus or plus 20 per cent. Their results showed that the titers of the mothers and the respective newborn infants were closely similar at birth; the infant's titer decreased up to the second year when it resumed the adult level where it remained until the sixth decade when a slight fall occurred.

In 1934, Parish, O'Meara, and Clark² described an antihemolysin titer technique by which 100 rabbits, 100 human beings, 50 horses, and 50 guinea pigs were tested. Since no standard antitoxin had been adopted, they chose to report their data in decimal fractions of a known antitoxin, which could later be linked with an official standard. Their results were as follows:

100 Rabbits	Titers from .003 to .03 of known K antiserum
100 Human beings	Titers from .003 to .03 of known K antiserum
50 Guinea pigs	Titers from .003 to .03 of known K antiserum
50 Horses	Titers from .003 to .07 of known K antiserum

McKie,³ in 1934, reported 36 cases of superficial staphylococcus infections on which antihemolysin titers were determined. No mention was made, however, of the technique or "units" employed.

From the Kansas City General Hospital, Kansas City, Mo., and the Department of Bacteriology, University of Kansas, Lawrence, Kan.
Received for publication, June 26, 1944.

based upon the fact that $\frac{1}{8}$ unit of antitoxin in 0.5 c.c. was used for standard, or the equivalent of $\frac{1}{4}$ unit in 1.0 c.c. Therefore the dilution was multiplied by $\frac{1}{4}$ or divided by 4.

RESULTS

In our group of 500 sera, only 10 or 20 were titrated at one time, because it was felt that accuracy was sacrificed when larger numbers were handled. If the end point appeared to be between adjacent dilutions, e.g., 1:2 and 1:4, the titer was read between the two, as 1:3. When the unit reading was below 1 unit, it was expressed in decimal fractions of 2 places, e.g., 1:3 dilution divided by 4 to give a 0.75 unit value.

Table I gives the results of the titer determinations, showing the number of sera with their respective international unit value.

DISCUSSION

In considering the use and relationship of antitoxin titers for diagnosis and therapy, the literature shows many studies on both animals and man.

Weiss¹¹ states that the rate of absorption, as measured by the blood serum content of antitoxin after intramuscular injection of antitoxin, is uniform in both monkeys and man. The amount of injected antitoxin corresponds uniformly with the serum determinations in cases using both small and large amounts of antitoxin.

The localization and concentration of antitoxin¹² in artificially produced areas of inflammation is significant and the use of counterirritants in the treatment of local infections is therefore indicated.

The resistance of rabbits to the intravenous injection of staphylococci suspensions is definitely correlated with the antitoxin content of their serum.¹³ Increased antitoxin titers decreased mortality rates from 80 per cent in normal titrated rabbits to 9 per cent in antitoxin-treated ones. Parish and Clark¹⁵ agree with these findings which certainly seem to justify more extensive human clinical use of antitoxin.

With histologic studies on mice immunized both actively and passively and then inoculated with staphylococci, the mechanism of immunity with antitoxin appears primarily as neutralization of the toxin.¹⁴ Leucocytes survive for longer periods in immune animals and continue to phagocytize the staphylococci more easily in presence of immune serum.

On human volunteers and patients with staphylococcal infections there has been considerable experimentation, employing staphylococcal toxoid to produce active immunization. Dolman,¹⁷ McKie,³ Murray,⁵ Blair and Hallman,²² Whitby,⁶ Buchman,¹⁰ Longacre,²³ and others have contributed in this field. In thirty-six cases of superficial staphylococcal infections, a rise in antihemolysin titer is accompanied by a clinical improvement.³

Amounts of antitoxin are not markedly different in normal persons and in those with chronic superficial lesions like furunculosis. There are varying results reported by different workers on the significance of diagnosis and therapy in chronic osteomyelitis. It is suggested that strains of staphylococci causing

these deep types of infections are of low toxigenicity and of high invasiveness, hence there is little correlation between the antitoxin titer and the progress of the disease.

In another series,⁶ toxoid therapy was found to be clinically effective in furunculosis, styes, carbuncles, but not in cases of pustular acne and syecosis.

An interesting comparison of antitoxin production in diabetic and normal children,⁸ showed delayed production and lowered maximum titers in diabetic children. There was also a correlation between the state of diabetic controls and antitoxin response: well-controlled diabetic cases responded similarly to normal children, and poorly controlled cases showed retardation and lowered production.

The value of passive immunity produced by the injection of antitoxin has been studied as a therapeutic agent. In 104 cases of staphylococcus infections treated with antitoxin,¹⁷ 24 out of 24 recovered from skin and subcutaneous infections; 22 out of 32 cases of staphylococcemia from osteomyelitis recovered; 5 out of 22 hopeless staphylococcemia in adults recovered. Other cases,¹⁸⁻²⁰ of staphylococcemia, and acute osteomyelitis showing favorable response to antitoxin therapy were cited. The fatality of staphylococcemia in 117 cases was found to be 91.4 per cent.²¹ In a study of 17 cases of the same type treated with staphylococcal antitoxin, Stookey and Searpellino reported a 53 per cent recovery. Early diagnosis and administration of antitoxin before the toxin gets fixed to the tissues are distinct aids in lowering the mortality rate.

The consensus of these various authors is that, when there is adequate drainage of pyogenic foci, staphylococcus antitoxin is a specific therapeutic agent of considerable usefulness. There is no contraindication to its use with other forms of therapy, such as blood transfusions, sulfonamides, or bacteriophage.

SUMMARY

A technique for determining staphylococcus antitoxin titers in blood serum by a hemolytic test has been described.

An average normal titer has been calculated from a series of 500 normal blood samples.

Reports are cited from the literature which indicate the value of direct antitoxin therapy or toxoid administration.

REFERENCES

1. Bryce, L., and Burnet, F. M.: Natural Immunity to Staphylococcal Toxins, *J. Path. & Bact.* 35: 183, 1932.
2. Parish, H. J., O'Meara, R. A., and Clark, W. H.: The Clinical Investigation of Staphylococcal Toxin, Toxoid, and Antitoxin, *Lancet* 1: 1054, 1934.
3. McKie, M.: Treatment of Superficial Staphylococcal Infection by Toxoid, *Brit. J. Dermat.* 46: 20, 1934.
4. Quarterly Bull., Health Organiz. of League of Nations, 6S, Jan., 1935.
5. Murray, D. S.: Staphylococcus Toxoid, *Lancet* 1: 303, 1935.
6. Whitby, L.: The Treatment of Staphylococcal Skin Lesions With Toxoid, *Lancet* 1: 1454, 1936.
7. Weiss, C.: A Study of Natural and Acquired Immunity to Staphylococcal Toxin in Monkeys, *J. Immunol.* 37: 185, 1939.
8. Bates, G., and Weiss, C.: Delayed Development of Antibody to Staphylococcus Toxin in Diabetic Children, *Am. J. Dis. Child.* 62: 346, 1941.
9. Blair, J. E., and Hallman, F. A.: Staphylococcal Antihemolysis in Osteomyelitis and Other Staphylococcal Infections, *Proc. Soc. Exper. Biol. & Med.* 33: 382, 1935.

10. Levine, B. S.: The Unity of the Hemolytic, Dermonecrotic, and Lethal Properties of Staphylococcal Exotoxin and of Their Corresponding Counterparts in Staphylococcal Antitoxin, *J. Path. & Bact.* 48: 291, 1939.
11. Weiss, C.: Absorption of Staphylococcal Antitoxin After Intramuscular Injection in Man and Monkeys, *Proc. Soc. Exper. Biol. & Med.* 43: 441, 1940.
12. Rigdon, R. H.: Localization and Concentration of Staphylococcus Antitoxin in Areas of Rabbit's Skin, *J. Lab. & Clin. Med.* 27: 37, 1941.
13. Smith, M. L.: Circulating Antitoxin and Resistance to Experimental Infection With Staphylococcus, *J. Path. & Bact.* 45: 367, 1947.
14. Rigdon, R. H.: Effects of Intraperitoneal Injections of Staphylococcus Antitoxin on Subcutaneous Staphylococcal Infection in Mice, *J. Lab. & Clin. Med.* 25: 251, 1939.
15. Parish, H. J., and Clark, W. H. M.: Staphylococcus Toxin and Antitoxin, *J. Path. & Bact.* 35: 251, 1935.
16. Buchman, J.: Use of Staphylococcus Toxoid in Treatment of Chronic Osteomyelitis, *J. A. M. A.* 108: 1151, 1937.
17. Dolman, C. E.: Staphylococcus Antitoxin Serum in the Treatment of Acute Staphylococcal Infections and Toxemias, *Canad. M. A. J.* 30: 691, 1934.
18. Parker, H.: The Treatment of Staphylococcal Septicemia, *J. Missouri M. A.* 35: 30, 1938.
19. Johnson, W.: Osteomyelitis With Staphylococcemia; Favorable Response to Antitoxin; Thrombophlebitis of Inferior Vena Cava, *South. Med. & Surg.* 100: 149, 1938.
20. Sager, W. W., Hunter, O. B., and Toktoi, G.: Staphylococcal Septicemia Treated With Staphylococcal Antitoxin, *M. Ann. District of Columbia* 9: 306, 1940.
21. Stookey, P. F., and Scarpellino, L. A.: Staphylococcal Septicemia, *South. M. J.* 32: 173, 1939.
22. Blair, J. E., and Hallman, F. A.: Staphylococcal Antihemolysin Titers Following Staphylococcal Toxoid in Chronic Osteomyelitis, *Proc. Soc. Exper. & Med.* 34: 637, 1936.
23. Longacre, A. B.: Studies of Antihemolysin Level in Patients With Staphylococcus Infections Treated With Staphylococcus Toxoid, *Surgery* 10: 576, 1941.
24. Hite, K., Banks, S., and Daack, G.: Studies on Bacteriology and Immunology of Chronic Staphylococcal Osteomyelitis. I. The Cultures Involved; II The Antihemolysin in the Patients' Serum; and the Local Inflammatory Reaction, *J. Infect. Dis.* 62: 317, 1938.

STUDIES ON THE TOXICITY OF GOLD COMPOUNDS IN RATS

CHARLES W. DENKO, PH.D.,* AND ARTHUR K. ANDERSON, PH.D.
STATE COLLEGE, PA.

THE reports of Møllgaard,¹ Forestier,² Towle and Grund³ Hartfall, Garland, and Goldie,⁴ Sabin and Warren,^{5, 6} and others have created considerable interest in the use of gold compounds for the treatment of tuberculosis, lupus erythematosus, and arthritis. However, the frequent, varied toxic reactions and occasional fatalities in patients receiving gold drugs have been a hindrance to their extensive use. In an effort to find a nontoxic, therapeutically effective drug, investigators have tested a large number of gold preparations without discovering one that is completely satisfactory.

In 1940 Sabin and Warren⁶ studied the effectiveness of gold compounds in curing an experimental mouse arthritis produced by inoculating mice with a pleuropneumonia-like organism. In their work they studied the toxicity of several gold compounds, using mice as test animals and using survival or death of the animal as an index of toxicity. Obviously this method gives no measure of the degree of toxicity in surviving animals.

In using a similar technique to test the toxicity of gold compounds in this laboratory, it was noted that mice receiving sublethal doses of gold salts exhibited an elevation of the nonprotein nitrogen level of the blood, often to two or three times normal values. From this it appeared that the nonprotein nitrogen value of the blood might be a better index of toxicity than survival or death. However, since a mouse had to be sacrificed to obtain sufficient blood for a nonprotein nitrogen determination, this method could hardly be considered satisfactory since it is often desirable to give an animal several doses of the drug. In order to eliminate the necessity of sacrificing an animal whenever its blood was to be analyzed, rats were used in the experiments reported here.

In the following report several gold compounds, some commercially available and others especially synthesized for this work, have been studied in order to obtain information with regard to their toxic reactions.

GOLD COMPOUNDS USED

Table I lists the gold compounds used in the following experiments together with their formulas and theoretical gold content. Especially prepared gold

*Present address: Research Laboratories, S. M. A. Corporation, Chagrin Falls, Ohio.
From the Department of Agricultural and Biological Chemistry, The Pennsylvania State College.

This paper is taken from a thesis presented to the Graduate School of the Pennsylvania State College by Charles W. Denko in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

Authorized for publication July 18, 1944, as paper No. 1239 in the journal series of the Pennsylvania Agricultural Experiment Station.

Received for publication, Aug. 5, 1944.

compounds were analyzed for gold as an index of purity. These values are given together with the method used for the preparation of each prepared compound.

TABLE I
GOLD COMPOUNDS USED

NAME	FORMULA	PER CENT OF GOLD		METHOD OF PREPARATION
		THEORETICAL	FOUND [§]	
1. " " " " " "	$C_2H_4O_4SAu$	59.		
2. " " " " " "	$Na_2Au(S_2O_3)_2 \cdot 2H_2O$	37.4		
3. " " " " " "				
4. " " " " " "	$Sr(OOCH_2SAu)_2$	59.56	60.61	(7), (8)
5. α -aurothio- <i>n</i> -butyric acid	$CH_3(CH_2)_3CH(COOH)SAu$	62.24	61.40	(7), (8)
6. Auro- <i>n</i> -propyl xanthate	$CH_3(CH_2)_2OCSSAu$	59.29	59.34	(9), (10)
7. Auro-isopropyl xanthate	$(CH_3)_2CHOCSSAu$	59.29	57.70	(9), (10)
8. Auro- <i>n</i> -butyl xanthate	$CH_3(CH_2)_3OCSSAu$	56.92	55.73	(9), (10)
9. Auro-isobutyl xanthate	$(CH_3)_2CHCH_2OCSSAu$	56.92	56.64	(9), (10)

*Supplied through the courtesy of the Schering Corporation, Bloomfield, N. J.

†Supplied through the courtesy of Mr. J. G. Bieri

‡Supplied through the courtesy of Crookes Laboratories, Inc., New York, N. Y.

§Method of the American Medical Association.¹¹

TOXICITY OF GOLD COMPOUNDS

The effects of gold compounds on the nonprotein nitrogen of the blood are given in Table II. The Anderson-Howell¹² method was used to determine all the nonprotein nitrogen values. Gold sodium thiosulfate was administered in water solution. The colloidal gold was a hydrosol. Water-insoluble strontium aurothioglycolate and the auroxanthates were administered intramuscularly in a sesame oil suspension. Since α -aurothio-*n*-butyric acid is soluble only to the extent of 54 mg. per 100 c.c. of water at 100° C., it also was administered in oil suspension. Gold sodium thiosulfate was injected as an aqueous solution.

Gold sodium thiosulfate is definitely toxic when administered in 5 mg. doses. In Animal 23 death occurred on the third day when the blood nonprotein nitrogen had reached a level of 248.1 mg. per 100 c.c. In Animal 24 the blood nonprotein nitrogen reached a level of 151 mg. on the fourth day but returned to normal on the seventh day. In animals receiving less than 5 mg. there was no appreciable rise in blood nonprotein nitrogen except in Animal 28, receiving 2 mg., where a value of 150.1 mg. was found on the second day after injection. This animal returned to normal on the third day.

α -Aurothio-*n*-butyric acid was toxic when administered at a 3 mg. level, one animal having a nonprotein nitrogen level of 339 mg. and dying the third day after injection. On the 5 mg. level one animal died the third day when the nonprotein nitrogen level had reached 185.4 mg. per 100 c.c. of blood. The three other animals receiving 5 mg. doses had high nonprotein nitrogen values the third day after injection. The fifth day after injection two of these were essentially normal, while the third had returned to normal the tenth day.

In the case of all the other compounds studied, there was no increase in nonprotein nitrogen level even after doses of 100 mg. in the cases of auro-*n*-butyl and auro-isobutyl xanthates.

TABLE II

SHOWING NONPROTEIN NITROGEN OF RAT BLOOD BEFORE AND AFTER INJECTING GOLD PREPARATIONS

ANIMAL	SEX	WEIGHT (G.M.)	DOSE (MG.)	NONPROTEIN NITROGEN IN BLOOD (MG. PER CENT)											
				BEFORE INJECTION	DAYS AFTER INJECTION										
					1	2	3	4	5	7	8	10	11	12	13
Gold sodium thiosulfate															
30	F	106	1	54.6	52.8	44.4	44.1			36.9					
29	F	139	1	39.1	37.1	46.2	36.6			43.2					
28	F	139	2	39.6	41.4	150.1	42.7			41.4					
27	M	167	2	39.4	46.5	45.6	53.2			48.6					
26	F	121	3	40.6	48.2	52.2	39.2			43.9					
25	F	113	3	41.0	42.8	44.1	45.3			45.3					
24	M	157	5	69.7	85.4	139.6	149.4	151.0		42.2					
23	M	114	5	43.9	136.4	156.2	248.1*								
α -aurothio- <i>n</i> -butyric acid															
35	F	169	3	42.4	96.3	175.6	339.0*								
32	M	185	3	38.1	35.5	34.3	47.4		37.9						
37	M	146	5	51.9	90.6	151.2	180.9		56.4			39.7			
36	F	167	5	51.1	95.9	92.9	131.6		37.3			43.9			
33	F	185	5	42.6	96.4	151.5	170.4		192.3			42.1			
31	M	211	5	40.4	131.7	176.4	185.4*								
Colloidal gold (Ruby)															
38	M	201	1.25	64.2	39.3	50.7		39.1							
39	F	177	2.25	42.4	36.3	37.8		41.2							
Strontium aurothioglycolate															
40	M	215	5	47.4	36.1	40.4	40.4								
41	M	202	14	33.4	43.0	39.9	41.4								
Auro- <i>n</i> -propyl xanthate															
44	F	141	5	35.9	35.9	35.6	34.0								
45	M	144	10	35.1	30.7	30.5	38.9								
46	M	241	15	46.4	33.6	48.3	34.4								
47	M	217	25	37.5	41.1	41.1	33.7								
Auro-isopropyl xanthate															
57	F	141	5	42.0	35.5	35.4	38.1	37.8							
56	M	233	10	36.7	39.3	47.1	36.1	43.9							
58	F	186	15	39.1	31.2	40.0	37.5	44.6							
59	M	238	25	37.1	40.7	40.4	40.4	37.4							
Auro- <i>n</i> -butyl xanthate															
48	M	174	10	41.6	30.7	37.0		36.9							
49	M	192	20	31.0	38.6	44.0		35.3							
50	F	190	50	40.8	40.0	40.3		51.5							
51	F	189	100	51.0	36.3	39.6		41.7				38.5	35.3	49.7	
Auro-isobutyl xanthate															
52	F	203	10	40.5	34.0	36.3	42.9		46.5						
53	M	245	20	42.3	39.7	36.0	39.7	35.7	41.3						
54	F	183	50	30.7	30.7	35.6	38.5	43.2		38.3	36.8				
55	M	240	100	31.5	31.0	36.9	36.3	34.9							

*Died.

The results of this experiment indicate that the toxicity of gold compounds depends upon their solubility in water. Gold sodium thiosulfate and α -aurothio-*n*-butyric acid, which are water soluble, are toxic, whereas colloidal gold, strontium aurothioglycolate, and the auroxanthates, water-insoluble substances, are nontoxic, as indicated by their effects on the nonprotein nitrogen values

of the blood. If a gold compound is toxic and the animal survives the dose given, the toxic reaction is temporary, the nonprotein nitrogen value of the blood returning to normal in from five to ten days after injection.

THE EFFECT OF TYPE OF CARRIER ON THE TOXICITY OF AUROTHIOGLUCOSE

Some of the compounds in the previous experiments were administered in oil suspensions and some were administered in aqueous solution. Many workers feel that administration of water-soluble aurothioglucose as an oil suspension makes this compound less toxic than when administered in aqueous solution. In order to determine the effect of type of carrier on the toxicity of aurothioglucose, animals were given intramuscular injections of varying doses in oil suspension and in aqueous solution. The effects of these injections on the nonprotein nitrogen level of the blood are given in Table III.

TABLE III
SHOWING THE EFFECT OF TYPE CARRIER ON THE TOXICITY OF AUROTHIOGLUCOSE

ANIMAL	SEX	WEIGHT (GM.)	DOSE (MG.)	CARRIER	NONPROTEIN NITROGEN IN BLOOD (MG. PER CENT)						
					BEFORE INJECTION	DAYS AFTER INJECTION					
						1	2	3	4	7	15
1	F	141	Control	0.9% NaCl	53.3	43.5	50.7				34.0
10	M	185	1.0 c.c. Control	Sesame oil		51.8	41.2	39.8		44.9	
2	M	161	10	Water	36.4	34.7	40.7	40.7		42.0	44.4
6	F	134	10	Oil	37.7	61.1	44.3	50.2		38.1	
4	F	143	40	Water	45.3	141.0	169.5	184.1		87.8	44.6
7	F	162	25	Oil	55.0	131.0	170.5	266.8*			
3	F	170	50	Water	51.8	101.0	197.5	151.5		81.2	44.2
8	M	180	50	Oil	60.0	126.0	214.2	205.6	363.6*		
5	M	184	100	Water	48.6	97.8	263.0	287.2	340.0*		
9	M	169	100	Oil	54.3	120.0	238.0	209.0		107.5	

*Died.

Table III shows a comparison of the effect of various doses of aurothioglucose on the nonprotein nitrogen level of the blood when administered in water solution and in oil suspension. There appears to be little difference in the toxicity of aurothioglucose as related to the mode of injection. On the 25 and 50 mg. levels, aurothioglucose appears to be more toxic when administered in oil than when administered in water solution, as indicated by the death of both animals receiving the oil suspension. On the 40 and 50 mg. levels in water solution both animals survived and were back to normal the fifteenth day. However, on the 100 mg. level death occurred in the animal receiving the water solution, whereas the animal receiving the oil suspension survived.

TOXICITY OF α -AUROTHIO-*n*-BUTYRIC ACID, DUE TO GOLD ATOM

In order to prove that the toxicity of gold compounds was due to the presence of gold, animals were given injections of α -thio-*n*-butyric acid and later α -aurothio-*n*-butyric acid. The effects of these compounds on the nonprotein nitrogen level of the blood are given in Table IV.

TABLE IV

TOXICITY OF α -AUROTHIO-*n*-BUTYRIC ACID DUE TO PRESENCE OF GOLD ATOM

ANI- MAL	SEX	WEIGHT (GM.)	α -THIO- <i>n</i> - BUTYRIC ACID (MG.)	NONPROTEIN NITROGEN IN BLOOD (MG. PER CENT)				α -AURO- THIO- <i>n</i> - BUTYRIC ACID (MG.)	NONPROTEIN NITROGEN IN BLOOD (MG. PER CENT)				
				BEFORE INJEC- TION	DAYS AFTER IN- JECTION				DAYS AFTER INJECTION				
					1.	2	3			1	2	3	5
31	M	201	50	36.9	41.3	47.8	40.4	5	131.7	176.4	185.4*		
32	M	181	25	48.4	41.4	38.1		3	35.5	34.3	47.4	37.9	
33	F	174	10	47.2	37.9	39.7	42.6	5	96.4	151.5	170.4	192.3	42.1
34	F	174	0.50 c.c. 0.9% NaCl	48.0	45.4	37.8	39.7	0.50 c.c. oil	41.4	42.3	42.3	43.9	

*Died.

Table IV indicates that α -thio-*n*-butyric acid, when administered in doses up to 50 mg., is nontoxic. When this is followed in the same animals by doses of 5 mg. of α -aurothio-*n*-butyric acid, definite toxicity results as indicated by a rise in blood nonprotein nitrogen values, and in one case by the death of the animal. It is evident from this that toxicity is due to gold.

DEVELOPMENT OF TOLERANCE TO AUROTHIOGLUCOSE AND GOLD SODIUM THIOSULFATE

Cortell and Richards^{13, 14} studied the development of tolerance to gold salts in rats, using the survival method. They showed that rats given sublethal doses of gold sodium thiosulfate and of gold sodium thiomalate could tolerate ordinarily lethal doses of these compounds subsequently administered. They also showed that a rat which had developed tolerance to gold sodium thiomalate was also tolerant to gold sodium thiosulfate.

The results presented in Table V show the effects of repeated doses of aurothioglucose and of gold sodium thiosulfate on blood nonprotein nitrogen values. Rats given doses of 40 and 50 mg. of aurothioglucose showed a decided rise in blood nonprotein nitrogen values which returned to normal the fifteenth day. When the doses were repeated on these animals, blood nonprotein nitrogen values remained normal. A third dose of 75 and 100 mg. also showed no appreciable rise in the blood nonprotein nitrogen value. Animal 2, given an initial dose of 10 mg., a second dose of 20 mg., and a final dose of 50 mg., showed little change in blood nonprotein nitrogen values.

The results obtained with gold sodium thiosulfate although similar to those obtained with aurothioglucose are not as consistent. Seven rats were given initial doses ranging from 1 to 5 mg. Only two animals showed a rise in blood nonprotein nitrogen values, the one receiving 2 mg. and the other 5 mg. As a second dose each animal was given 5 mg. Apparently an initial dose of 1 mg. did not protect the animal since both of these animals died after the 5 mg. dose. Even initial doses of 2 and 3 mg. showed slight protection since in all cases the blood nonprotein nitrogen values were high, and in Animal 26, which had received an initial dose of 3 mg., death occurred. An initial dose of 5 mg. apparently protected the animal because there was little rise in the blood nonprotein nitrogen value after the second dose of 5 mg.

The four surviving animals were each finally given a 10 mg. dose. All animals survived and blood nonprotein nitrogen values were approximately normal on the seventh day after injection except in Animal 28. Animal 28

TABLE V

SHOWING DEVELOPMENT OF TOLERANCE TO AUROTHIOGLUCOSE AND GOLD SODIUM THIOSULFATE

ANIMAL	SEX	WEIGHT GM.		NONPROTEIN NITROGEN IN BLOOD (MG. PER CENT)						
				BE- FORE INJEC- TION	DAYS AFTER INJECTION					
					1	2	3	4	7	9
AUROTHIOGLUCOSE										
1st Dose										
			mg.							
1	F	141	1 c.c. NaCl	53.3	43.5	50.7				34.6
2	M	161	10	36.4	54.7	40.7	40.7		42.9	44.4
3	F	143	40	45.3	141.0	169.5	184.1		87.8	44.6
4	F	170	50	51.8	101.0	197.5	151.5		81.2	44.2
2nd Dose										
			mg.							
1		150	0.5 c.c. NaCl		60.8	49.4	51.1			56.4
2		212	20		47.1	41.7	53.0			52.4
3		155	40		49.8	39.1	44.2			49.9
4		175	50		44.7	42.2	48.4			53.8
3rd Dose										
			mg.							
1		168	1 c.c. NaCl		48.7	50.7				
2		221	50		51.9	44.8	54.6			
3		159	75		48.9	41.6	47.5			
4		187	100		50.7	47.4	52.4			
GOLD SODIUM THIOSULFATE										
1st Dose										
			mg.							
29	F	139	1	39.1	37.1	46.2	36.6		43.2	
30	F	106	1	54.6	52.8	44.4	44.1		36.9	
27	M	167	2	39.4	46.5	45.6	53.2		48.6	
28	F	139	2	39.6	41.4	150.1	42.7		41.4	
25	F	113	3	41.0	42.8	44.1	45.2		45.3	
26	F	121	3	40.6	48.2	52.2	39.2		43.9	
24	M	157	5	69.7	85.4	139.6	149.4		42.2	
2nd Dose										
			mg.							
29		149	5		150.0	207.2	280.0*			
30		126	5		94.2	174.0	240.0*			
27		167	5		123.6	42.4	54.5		49.4	
28		139	5		104.8	116.2	153.8		100.0	
25		133	5		117.8	119.5	100.0		84.8	
26		141	5		86.0	209.0	175.6	255.6*		
24		157	5		78.3	47.1	39.4		49.8	
3rd Dose										
			mg.							
27		214	10		92.7	78.9	63.1		55.7	
28		146	10		137.4	197.8	255.4		159.6	
25		138	10		162.4	95.0	63.1		48.1	
24		193	10		105.2	132.2	85.0		44.1	

*Died.

appears to have been exceptionally sensitive to gold since the initial dose of 2 mg. produced an appreciable rise in the blood nonprotein nitrogen value.

The results of these experiments indicate that rats become more tolerant to gold compounds after having received sufficiently large initial doses of them.

DEVELOPMENT OF CROSS-TOLERANCE

Animals that had received aurothioglucose were injected with gold sodium thiosulfate and later with α -aurothio-*n*-butyric acid. Animals that had re-

ceived gold sodium thiosulfate were injected with aurothioglucose and later with α -aurothio-*n*-butyric acid. Animals that had received auroxanthates were subsequently injected with aurothioglucose. The changes in the nonprotein nitrogen values of the blood following these injections are presented in Table VI.

TABLE VI
CROSS-TOLERANCE STUDIES

ANIMAL	SEX	WEIGHT (GM.)	GOLD SODIUM THIOSULFATE (MG.)	NONPROTEIN NITROGEN IN BLOOD (MG. PER CENT)					α -AUROTHIO- <i>n</i> -BUTYRIC ACID (MG.)	NONPROTEIN NITROGEN IN BLOOD (MG. PER CENT)				
				BEFORE INJECTION	DAYS AFTER INJECTION					DAYS AFTER INJECTION				
					1	2	3	5			9	1	2	3
Aurothioglucose*-gold sodium thiosulfate- α aurothio- <i>n</i> -butyric acid														
3	F	181	10	52.4	98.0	76.9	49.6		42.7	10	62.1	41.8	44.6	40.0
Gold sodium thiosulfate†-aurothioglucose- α -aurothio- <i>n</i> -butyric acid														
			Aurothio-glucose (mg.)											
27	M	183	100	55.7		49.9	87.3	50.0		10	61.1	55.8	43.1	
Auro- <i>n</i> -propyl xanthate†-aurothioglucose														
45	M	158	100	34.0	38.2	42.1	39.9	42.8						
Auro- <i>n</i> -butyl xanthate†-aurothioglucose														
49	M	214	50	35.3	50.6	51.7	56.6							
Auro-isobutyl xanthate†-aurothioglucose														
52	F	213	100	46.5	49.3	58.3								
Auro-isopropyl xanthate†-aurothioglucose														
58	F	179	50	44.6	77.0	112.8	125.0							
Controls—aurothioglucose														
64	M	186	50	49.4	102.3	132.4								
62	M	257	100	39.3	164.8	170.5								

*See Table II for effects of original doses.

†See Table I for effects of original doses.

Animal 3, which had originally received a dose of 50 mg. of aurothioglucose and whose blood nonprotein nitrogen value had reached 197.5 mg. per cent two days after injection, tolerated a 10 mg. dose of gold sodium thiosulfate. The blood nonprotein nitrogen value reached 98.0 mg. per cent one day after the gold sodium thiosulfate was given. Later this animal tolerated a 10 mg. dose of α -aurothio-*n*-butyric acid with very little rise in the blood nonprotein nitrogen value. Ten milligram doses of gold sodium thiosulfate and α -auro-*n*-butyric acid given to a normal rat are usually lethal.

Animal 27 originally received 2 mg. of gold sodium thiosulfate with little subsequent rise in blood nonprotein nitrogen values. When later given 100 mg. of aurothioglucose, the blood nonprotein nitrogen value rose to 87.3 mg. per cent three days after injection. Later this animal tolerated 10 mg. of α -aurothio-*n*-butyric acid with little rise in blood nonprotein nitrogen values.

As indicated in Table II, auroxanthates, even when given in 100 mg. doses, showed no toxicity as indicated by blood nonprotein nitrogen values. However, animals which had received auroxanthates show tolerance for subsequently administered aurothioglucose, as indicated in Table VI. Whereas

50 and 100 mg. doses of aurothioglucose show marked rises of blood nonprotein nitrogen values when given to normal animals, very little rise in blood nonprotein nitrogen values is noted when these doses are given to animals previously given auroxanthates. Of the auroxanthates studied, auro-isopropyl xanthate appears to be the only one that did not protect the animal against the toxic effect of subsequently administered aurothioglucose. Although the therapeutic value of auroxanthates has not been demonstrated, their effect in desensitizing animals is clearly demonstrated.

HISTOPATHOLOGIC STUDIES

Cortell and Richards¹⁴ have found severe kidney damage in rats receiving gold sodium thiosulfate. Brown, Saleeby, and Schamberg,¹⁵ in a study on rabbits, reported that marked injury to the kidneys may take place before definite blood changes occur. Block, Buchanan, and Freyberg^{16, 17} have reported finding gold in the kidneys and other organs of the body following administration of various gold drugs.

In the studies here reported, autopsies were performed on all animals. Histopathologic studies of the kidney were made on several representative animals by Dr. W. T. S. Thorp, of the Animal Pathology Laboratory.

Insoluble gold compounds were found largely unabsorbed at the site of injection for as long as two weeks after administration. No evidence of the presence of the soluble compounds was noted. Nephritis was noted in most cases studied histologically. In all cases of nephritis a high blood nonprotein nitrogen value was noted. However, kidney damage was not observed in all cases where a high nonprotein nitrogen value occurred. From this it would appear that blood nonprotein nitrogen values serve as a more sensitive index of toxicity than the histologic study of the kidney.

SUMMARY

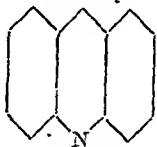
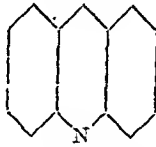
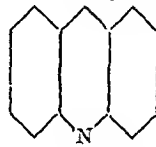
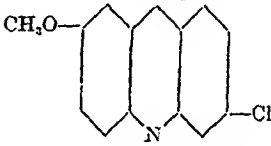
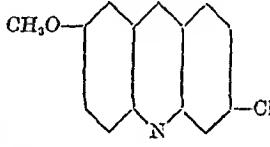
1. The nonprotein nitrogen value of the blood has been used in determining the toxicities of several commercial and experimentally synthesized gold compounds.
2. The water-soluble compounds, gold sodium thiosulfate, aurothioglucose, and slightly soluble α -aurothio-*n*-butyric acid, were found to be toxic.
3. The water-insoluble compounds, colloidal gold and auro-*n*-propyl, auro-isopropyl, auro-*n*-butyl and auro-isobutyl xanthates, were found to be nontoxic.
4. The toxicity of α -aurothio-*n*-butyric acid was found to be due to the gold portion of the molecule.
5. Tolerance and cross-tolerance to gold compounds were developed in animals.
6. High blood nonprotein values were always found where tissue changes indicating kidney damage had occurred. In some cases high blood nonprotein nitrogen values occurred where there was no evidence of tissue change.

REFERENCES

1. Møllgaard, H.: *Chemotherapy of Tuberculosis*, Copenhagen, 1924, Nyt Nordisk Forlag.
2. Forestier, Jacques: *Rheumatoid Arthritis and Its Treatment by Gold Salts*, J. LAB. & CLIN. MED. 20: 827, 1935.
3. Towle, H., and Grund, J.: *Progress in Dermatology*, New England J. Med. 202: 231, 1930.

4. Hartfall, S. J., Garland, H. G., and Goldie, Wm.: Gold Treatment of Arthritis, *Lancet* 233: 784, 1937.
5. Sabin, A. B., and Warren, Joel: The Therapeutic Effectiveness of a Practically Nontoxic New Compound (Calcium Aurothiomalate) in Experimental, Proliferative Chronic Arthritis of Mice, *Science* 92: 535, 1940.
6. Sabin, A. B., and Warren, Joel: The Curative Effect of Certain Gold Compounds on Experimental Proliferative Chronic Arthritis in Mice, *J. Bact.* 40: 823, 1940.
7. Biilman, Einar: Studien über organische Thiosäuren, I, *Ann.* 339: 351, 1905.
8. Biilman, Einar: Studien über organische Thiosäuren, II, *Ann.* 348: 120, 1906.
9. Zeise, W. C.: Beiträge zur nahen Kenntnis und ihrer Verbindungen, *Ann.* 16: 178, 1835.
10. Scala, A.: Su alcuni derivati dell' acido propilxantogenico, *Gazz. Chim. Ital.* 17: 78, 1887.
11. American Medical Association: New and Nonofficial Remedies, 1940, Chicago, American Medical Association.
12. Anderson, Arthur K., and Howell, Stacey F.: The Determination of Nonprotein Nitrogen in One-Tenth of a Cubic Centimeter of Blood, *J. LAB. & CLIN. MED.* 16: 183, 1930.
13. Cortell, Ruth, and Richards, R. K.: Development of Tolerance to Gold Salts in Rats, *Proc. Soc. Exper. Biol. & Med.* 49: 121, 1942.
14. Cortell, Ruth, and Richards, R. K.: Studies on the Tolerance Formation to Gold Sodium Thiosulfate in Rats, *J. Pharmacol. & Exper. Therap.* 76: 17, 1942.
15. Brown, H., Saleeby, E. R., and Schamberg, J. F.: Study of Toxic Effects of Certain Gold Compounds as Indicated by the Blood Chemistry and Pathological Changes in the Organs, *J. Pharmacol. & Exper. Therap.* 28: 141, 1926.
16. Block, W. D., Buchanan, O. H., and Freyberg, R. H.: Metabolism, Toxicity and Manner of Action of Gold Compounds Used in the Treatment of Arthritis, II, *J. Pharmacol. & Exper. Therap.* 73: 200, 1941.
17. Block, W. D., Buchanan, O. H., and Freyberg, R. H.: Metabolism, Toxicity, and Manner of Action of Gold Compounds Used in the Treatment of Arthritis, IV, *J. Pharmacol. & Exper. Therap.* 76: 355, 1942.

TABLE I

COMPOUND	MOLECULAR WEIGHT	STRUCTURAL FORMULA
9-Aminoacridine	194.2	NH_2 
9-Aminoacridine hydrochloride	230.7	$\text{NH}_2 \cdot \text{HCl}$ 
9-Aminoacridine sulfamate	291.3	$\text{NH}_2 \cdot \text{HOSO}_2\text{NH}_2$ 
3-Chloro-7-methoxy-9-aminoacridine hydrochloride	295.2	$\text{NH}_2 \cdot \text{HCl}$ 
3-Chloro-7-methoxy-9-(4-diethylaminobutylamino)-acridine dihydrochloride trihydrate	512.9	$\text{HNCH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{N}(\text{C}_2\text{H}_5)_2$  $\cdot 2\text{HCl} \cdot 3\text{H}_2\text{O}$

utes. After cooling, normal sterile horse serum was added to the tubes containing the veal-dextrose broth to a concentration of 1 per cent.

Preliminary solubility studies showed 9-aminoacridine and 3-chloro-7-methoxy-9-aminoacridine hydrochloride to be but slightly soluble in water. These compounds were dissolved, therefore, in a few c.c. of ethyl alcohol preparatory to making the initial broth dilution. The greatest concentration in which the latter compound would remain in solution completely was 1:8,000.

A preliminary test on an aqueous solution of 3-chloro-7-methoxy-9-(4-diethylaminobutylamino)-acridine dihydrochloride trihydrate revealed that it lost approximately 50 per cent of its activity upon autoclaving. For this reason a 1 per cent aqueous solution of the compound was sterilized by Berkefeld filtration. Subsequent serial dilutions from the stock solution were prepared aseptically in sterile broth.

The highest drug concentration of all the acridines studied did not alter the original pH of the broth media. Suitable control demonstrated that the small

TABLE II
HIGHEST DILUTION OF COMPOUND EXHIBITING ANTIBACTERIAL ACTIVITY

	9-AMINO-ACRIDINE		9-AMINO-ACRIDINE HCl		9-AMINO-ACRIDINE SULFAMATE		3-CHLORO-7-METHOXY-9-AMINO-ACRIDINE HCl		3-CHLORO-7-METHOXY-9-(4-DIETHYLAMINO)BUTYL-AMINO-ACRIDINE 2HCl·3H ₂ O	
	Bs	Bc	Bs	Bc	Bs	Bc	Bs	Bc	Bs	Bc
Pneumococcus Type I	250,000	256,000	250,000	128,000	128,000	128,000	256,000	256,000	256,000	128,000
Pneumococcus Type II	256,000	128,000	256,000	128,000	128,000	128,000	512,000	256,000	512,000	256,000
Pneumococcus Type III	128,000	128,000	16,000	2,000	16,000	4,000	256,000	128,000	512,000	256,000
Streptococcus Hemo-lyticus	128,000	128,000	128,000	128,000	128,000	32,000	128,000	128,000	256,000	64,000
Streptococcus Viridans	64,000	8,000	64,000	32,000	32,000	8,000	128,000	128,000	64,000	32,000
Streptococcus Agalactiae	128,000	128,000	128,000	128,000	128,000	32,000	128,000	128,000	128,000	16,000
Staphylococcus Aureus	64,000	8,000	32,000	8,000	32,000	8,000	64,000	64,000	64,000	4,000
Bacillus pyocyaneus	64,000	2,000	64,000	2,000	64,000	1,000	<8,000	<8,000	1,000	<1,000
Clostridium Welchii	128,000	128,000	128,000	128,000	128,000	64,000	256,000	256,000	1,024,000	256,000
Clostridium Tetani	128,000	128,000	128,000	128,000	128,000	128,000	256,000	128,000	1,024,000	256,000
Clostridium histolyticus	256,000	128,000	256,000	32,000	128,000	32,000	512,000	8,000	2,048,000	32,000
Clostridium Septicum	256,000	128,000	128,000	128,000	128,000	128,000	512,000	256,000	2,048,000	512,000

Bs = Bacteriostatic.

Bc = Bactericidal.

<8,000 = This compound insoluble in concentrations greater than 1:8,000, hence could not be tested.
 <1,000 = Concentrations greater than 1:1,000 not tested.

LABORATORY METHODS

GENERAL

FACTORS INFLUENCING THE ROMANOVSKY STAINING OF BLOOD FILMS AND THE ROLE OF METHYLENE VIOLET

R. D. LILLIE, M.D.*
BETHESDA, MD.

THE capriciousness of Wright's stain and its variations in staining from one sample to another have long been a matter of concern to hematologists. Many variations in technique of using the stain have been suggested, but it has still been necessary to determine the behavior of each new sample before consistent results could be obtained. Some samples do better with relatively longer "fixation" time before dilution, some with shorter staining time, and some with longer staining time after dilution. Some tolerate greater dilutions and give superior performance with them; others are ineffective under these conditions.

There are, furthermore, considerable variations in the color values attained. Samples resembling Jenner's stain give from light to fairly deep blue nuclei, blue lymphocyte cytoplasm, and good neutrophile and eosinophile granule stains but fail to stain the chromatin of blood protozoa. Then there are stains giving from deep violet to reddish purple chromatin, blue lymphocyte cytoplasm, perhaps less brilliant eosinophile granules, and good neutrophiles. A third group is recognized which gives deep red-purple chromatin, from gray to lilac lymphocyte cytoplasm, and less well-stained eosinophile granules. Generally speaking, stains of the second class are preferable both for blood morphology and for parasite identification; the third class will serve and the first group is inferior.

The color of the red corpuscles can vary from gray-green to bright orange-pink, but it is now generally recognized that any desired shade may be attained by adjustment of the pH of the diluting water. For instance, pH 7.0 gives gray or faintly orange-pink corpuscles, pH 6.8 more pink, 6.2 to 6.0 bright pink with decrease in intensity of nuclear staining; 6.5 or 6.4 is preferred by many.

The color variations in nuclear and cytoplasmic staining are fairly clearly associated with the composition of the basic component of the Romanovsky stain. Azure A tends to give deep red-purple chromatin and gray cytoplasm.^{1, 2} Samples in which azure A predominates give absorption spectra with maxima

*Senior Surgeon, U. S. Public Health Service.

From the Pathology Laboratory, National Institute of Health.

Received for publication, Aug. 29, 1941.

around 620 to 630 $m\mu$. Azure B gives blue cytoplasm, a more violet-purple chromatin stain, and an absorption maximum around 645 $m\mu$. An increase in the proportion of methylene blue alters the color of chromatin staining progressively toward deep blue, and as the amount of azure further decreases, the characteristic light blue nuclear staining of Jenner's stain is reached; the absorption maximum then approaches 665 $m\mu$.

The problem of qualitative composition of a Romanovsky stain is primarily one for the manufacturer rather than for the hematologic technician, since few users now manufacture their own stain. In the past, very wide variations have occurred in successive samples from the same manufacturer. Successive batches from manufacturer C gave absorption band medians at 658, 654, and 621, 655 and 657, 654 and 656, 657, 657, and 659 $m\mu$; from manufacturer A, at 618, 655, and 654; from manufacturer B, at 624 and 621; from manufacturer L, at 658 and 660, 660, 659, 661, 656, 654, 655, 652, and 657, 655 and 657, 655, 656, and 658 and 659; and from manufacturer N, at 631 and 633, 640 and 655, 638 and 650, 634 and 643, 653 and 628, 652 and 656, 634 and 638, 633 and 657, 646 and 652, 654 and 657, and 652. Attempts to make samples in the laboratory by Wright's method gave even wider variation in results: 618, 618, 651, and 629 $m\mu$. Spectrographic control aids in attaining greater uniformity, and greater uniformity has been noted in the more recent commercial samples since the suggestion that it be used was published in 1943.³

The selection of the proper range of composition for a Romanovsky stain for clinical laboratory use is not entirely a matter of personal preference in color values, as might be assumed from the above. It has been shown^{4, 5} that the stock solutions of the eosinates of methylene blue and the azures in methanol, methanol + glycerol, and other solvents undergo progressive alteration in the direction of formation of lower azures and methylene violets. This change is faster in some solvents than others, perhaps as fast in methanol as in any, slowest in equal volume mixtures of glycerol-methanol and glycerol-ethanol. It is accelerated markedly by small amounts of alkali in methanol, retarded by acid. The presence of acid, however, disturbs the eosin-thiazin balance, so that on dilution with water the eosin is thrown out as color acid. It further appeared that thiazin eosinates composed chiefly of methylene blue and azure B eosinates remained usable far longer after solution in methanol than those in which azure A was the dominant thiazin component.

Since continued experience showed that thiazin eosinates made by the Wright⁶ process continued to give results which varied from lot to lot, it was determined to explore the possibility of substituting MacNeal's^{7, 8} acid chromate process as modified by Holmes and French⁹ and by me^{10, 11} for making azure B eosinates for the traditional sodium bicarbonate process. This latter is, properly speaking, a sodium carbonate process, since the reaction is carried out in Wright's method at about 95 to 98° C., well above the 80° C. point, at which NaHCO_3 breaks down to Na_2CO_3 and CO_2 .

Since it had been found previously¹⁰ that medicinal methylene blue of 85 to 90 per cent dye content required about 250 mg. of $\text{K}_2\text{Cr}_2\text{O}_7$ per gram to convert it to a crude azure B (absorption band median about 647) and that 100 mg. gave an absorption band median about 658, it was determined to make a series of polychrome methylene blue samples by boiling 5 Gm. lots of methy-

lene blue twenty minutes in 300 c.c. of approximately 0.2 M^{*} sulphuric acid with 150 mg. potassium bichromate per gram dye. These were then cooled to 10° C. and neutralized by the cautious addition of sodium bicarbonate in precalculated amount (10.5 Gm., a little in excess of the 10.08 Gm. theoretically required). To each portion was then added 80 c.c. of 5 per cent eosin Y (NE-19) with constant shaking. After some hours the precipitates were removed by filtration through a Buchner funnel, rinsed with two 50 c.c. portions of distilled water and one 25 c.c. portion of 95 per cent alcohol. Precipitates were then dried on a warm plate at about 35° C. Eight lots of methylene blue from three manufacturers were thus used: NA-15, NA-19, NA-24, LA-7, LA-11, CA-24, and CB No. 430835 and No. 480843.

As the first of the eight was precipitated in two fractions in determining just how much eosin to use, there resulted nine lots of eosinate. As estimated from the absorption spectra in accordance with previously published tables,¹² these ranged from 21 to 28 per cent azure B to from 79 to 72 per cent methylene blue as eosinate. The medians of the absorption bands² were 656 and 659 m μ for the divided lot: 658, 658, 658, 658, 660, 569, and 659, respectively, for the nine lots.

These were severally dissolved in methanol at 150 mg. per 100 c.c. After three days with periodic shaking these were tested by staining methanol-fixed rat blood films containing numerous *Trypanosoma equiperdum*. Mixtures of stain and of water buffered to pH 6.5 in proportion of 1:2 (measured in cubic centimeters with graduated serologic pipettes, not by drops) were made and films were stained 3 and 5 minutes on the slide. Blue-violet to violet-purple nuclei and blue lymphocyte cytoplasm were obtained with all, and all nine stains were deemed satisfactory. Samples of the dry stain powders were submitted also to two other observers. One of these used Wright's¹³ earlier directions of 300 mg. dry stain per 100 c.c. methanol and obtained quite satisfactory results with all samples tried. The other used Conn's¹⁴ directions of 100 mg. in 60 c.c. methanol, and a drop-for-drop dilution with water at pH 6.5, and obtained quite variable results. Two were considered very good; 3, good; 2, fair; and 2, poor. However, a second series of six samples from a single lot of polychrome methylene blue eosinate submitted to this same observer under code gave equally discordant results: 2, very good; 1, good; 2, fair; and 1, poor. Hence the variability of this observer's results would seem to be due to factors other than the dye itself. Discussion of possible factors involved will be reserved for a later point.

To determine whether a less blue tone for nuclear chromatin would be desirable, a second series of polychrome methylene blues were made by the same method, except that the potassium bichromate was increased to 200 mg. per gram methylene blue, and eosinates were prepared as before. Seven lots of medicinal methylene blue and one of the double zinc chloride were used: NA-24, M No. 41930, M No. 32718, NA-14, NA-8, NA-12, CA-21, and NA No. 9781 (ZnCl₂). Medians of the thiazin absorption bands fell at 653, 651, 651, 651, 651, 649, 650 and 651. Methanol solutions were made at 300 mg. per 100 c.c.

*1.16 per cent by volume of concentrated acid, specific gravity 1.84, 95.5 per cent.

[†]The word purple is used in this paper to designate a color between red and spectral violet.

by shaking at intervals for three days. Three-minute stains on the slide with mixtures of 1 part (0.8 c.c.) stain and 2 parts (1.6 c.c.) water buffered to pH 6.5 gave uniformly excellent staining of leucocytes and trypanosomes. Dilutions of 1 part (4 c.c.) stain to 9 parts (36 c.c.) buffered water in a coplin jar gave similarly uniformly good stains in 20 minutes.

Another series of eosinates was made by the same technique from five samples of methylene blue furnished by the National Aniline Division, four of the ZnCl_2 double salt (methylene blue BB) and one medicinal, using again 200 mg. $\text{K}_2\text{Cr}_2\text{O}_7$ per gram. The methylene blue BB samples yielded eosinates with absorption band medians at 649, 647, 648, and 646, approximately in the same range as medicinal methylene blue samples heated with 250 mg. $\text{K}_2\text{Cr}_2\text{O}_7$ per gram.¹⁰ The medicinal methylene blue gave a median at 650.5, agreeing with the previous set.

Stock solutions of all were made in equal volumes of glycerol and methanol at 600 mg. per 100 c.c., and rat trypanosomiasis films were first fixed in methanol and then stained for varying periods in a 1:25 dilution in water buffered to pH 6.5. With all five samples, leucocytes were overstained and excellent details of trypanosome morphology obtained with an hour's staining; 30 minutes gave satisfactory, though less intense, staining of trypanosomes and excellent blood cell staining in all. Blood cells were quite well stained in from 10 to 15 minutes. These samples, made with no special precautions from the commercial methylene blue BB ($2\text{C}_{16}\text{H}_{18}\text{N}_3\text{SCl} \cdot \text{ZnCl}_2 \cdot \text{H}_2\text{O}$), were in no way inferior to those made from medicinal methylene blue ($\text{C}_{16}\text{H}_{18}\text{N}_3\text{SCl}$), the only difference being due to the lower dye content consequent on the presence of 1 molecule of ZnCl_2 (MW:136.3) and 1 molecule of water (MW:18) for each 2 molecules of methylene blue (MW:319.7), a proportion of 80.5 per cent methylene blue. The chemical data are derived from the *Colour Index*.¹⁵

It was previously observed on several occasions that Romanovsky staining was unsatisfactory in coplin jars when the same proportions of stock methanol solution and buffered water were used as give satisfactory stains on the slide. Hence, it was decided to explore further the limitations imposed by the final concentration of methanol in the diluted stain.

Two lots of ploychrome methylene blue eosinate, both made by the foregoing acid chromate process, were used in stock methanol solution, 44 D at 300 mg. per 100 c.c.; the other, 44 B, at 150 mg. per 100 c.c. Rat trypanosomiasis blood films were previously fixed from 2 to 3 minutes in methanol and stained in coplin jars for varying intervals. Into each of seven jars were placed 4 c.c. of stock stain. In the first, 36 c.c. water were added; in the second, 1 c.c. methanol and 35 c.c. water; in the rest, 2, 3, 4, 5, and 6 c.c. methanol with water to make 40 c.c. total as before. This gave final methanol percentages ranging by 2.5 per cent steps from 10 per cent to 25 per cent. All jars were covered during staining. With both samples placed in the first and second jars (at 10 and 12.5 per cent methanol), excellent and good stainings were attained respectively in 1 and 2 hours, quite good and fair in 30 minutes. The same grade of staining with each sample was attained in 2 hours at 15 per cent methanol, but staining in 1 hour was inferior. Fair staining only could be obtained in 2 hours at 17.5 per cent methanol, and in from 20 to 25 per cent methanol, nuclei remained pale blue even with 2 hours' staining.

It would appear that the upper limit for methanol concentration in coplin jar staining was between 12.5 and 15 per cent.

In the last series the stock solution containing 300 mg. per 100 c.c. gave consistently better results than that containing only 150 mg. per 100 c.c. This indicates that the final concentration of 0.15 mg. per cubic centimeter is too low to be effective in from 1 to 2 hours' staining, while 0.3 mg. per cubic centimeter gives good results in 30 minutes and excellent in 1 hour.

Since the concentrations of methanol in staining blood films in the usual manner are initially far higher than those which permit staining in a covered jar, a series of tests was made to see whether prevention of evaporation or its acceleration would influence staining on the slide. Dilutions were 1 part (0.8 c.c.) of stain and 2 parts (1.6 c.c.) of water and 1 (0.6 c.c.) of stain and 3 (1.8 c.c.) of water, buffered as usual at pH 6.5. Stains done in the open with or without an electric fan blowing were satisfactory, but when staining was done in the same manner under a bell jar, where the atmosphere was previously saturated with methanol vapor, cell nuclei remained pale blue.

Another series was done at a 1 part stain to 3 parts water dilution of another acid chromate (200 mg. per gram), polychrome methylene blue eosinate (44F). Rat trypanosomiasis blood films were first fixed in methanol for from 2 to 3 minutes immediately after making. Then they were stained 2, 3, 5, and 8 minutes. In one series, 0.5 c.c. stain (300 mg. per 100 c.c.) was deposited on the slide, and 1 minute later, 1.5 c.c. water buffered to pH 6.5 were added, while in another series stain and buffered water were first mixed in a test tube and then deposited on the slide. This second series generally showed better and more uniform staining than the first.

In some of those preparations mixed on the slide, mixing was accomplished by rocking the slide, in some by a gentle air stream, both moist and dry air being tried. Little difference was apparent among these. Some films were stained in quiet air with doors and windows closed; others, with an electric fan blowing directly across the staining rack. This again made little evident difference. However, placing the films on a warm plate at 37° C. during staining accelerated it somewhat and definitely increased the intensity. One repetition of the methanol moist chamber experiment gave only pale blue nuclei; another gave fairly good results. This chamber was not designed to maintain saturation particularly well, so the one successful stain of the three trials is probably significant rather of a lowered methanol vapor content of the air in the chamber than of a negation of the other two experiments.

Since sharper nuclear staining and more vivid color contrasts are obtainable by Giemsa type stains than by the usual Wright stain, it was determined to try this lot 44F also in glycerol-methanol stock solution at 1:24 and 1:49 dilutions. To estimate the proper dye content for such a stock solution, 50 c.c. quantities of an equal volume mixture of methanol and glycerol were placed in each of twelve bottles and to these were added, respectively, 1.0, 0.9, 0.8, 0.7, 0.6, 0.5, 0.4, 0.3, 0.25, 0.2, 0.15, and 0.1 Gm. of polychrome methylene blue eosinate 44F. When dilution (d) \times optical density (D) at both the absorption maxima, thiazin and eosin, were plotted against milligrams per cubic centimeter of dry dye placed in the solvent, graphs were obtained which formed straight lines from 0 to 8 mg. per cubic centimeter. At 8 mg. per cubic centi-

meter, $d \times D = 580$ for the thiazin, 520 for the eosin. Beyond those points, $d \times D$ values were irregular, and less than predicted from the graph of lower values, reaching maxima of 764 and 705, respectively. Assuming that these maxima represent saturation values, the straight line graphs intersect the horizontals for these saturation values at 10.9- and 10.9+ mg. per cubic centimeter, respectively, for the thiazin and eosin graphs, a very close agreement. It would seem to be indicated from these figures that at the temperatures of 25° C. then prevailing, equal volumes of glycerol and methanol will dissolve nearly 11 mg. of this polychrome methylene blue eosinate per cubic centimeter of solvent.

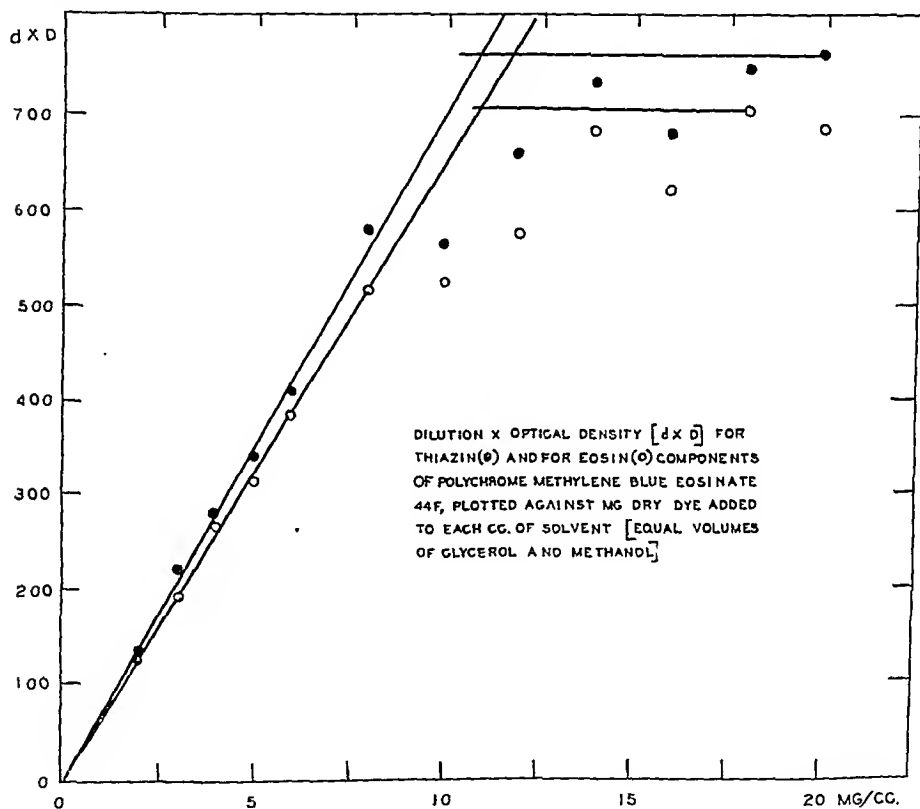


Fig. 1.

All of these stains were tested by staining rat trypanosomiasis blood films in eoplin jars at 1:49 dilution at pH 6.5 for varying periods. The solutions in which 0.8 per cent dye or more was used all gave excellent results for both leucocytes and trypanosomes in 30 minutes. The three with thiazin dilution \times density ($d \times D$) values above 700 gave excellent results in 10 minutes; the two with $d \times D$ values between 600 and 700 required 15 minutes; those with $d \times D$ between 500 and 600 required from 20 to 30 minutes.

With concentrations from 0.8 per cent down, the $d \times D$ values corresponded to the amount of dye added. The 0.6 per cent solution required 30 minutes at 1:24 dilution or 1 hour at 1:49 for excellent results; the 0.5 per

cent the same; the 0.4 per cent, 4 hours at 1:49 or 30 minutes at 1:24; the 0.3 per cent was ineffective at 1:49, but stained well at 1:24 in from 1 to 2 hours; and the 0.2 per cent was inferior in 2 hours at 1:24 but gave excellent results at 1:9 in 20 minutes.

It has been observed that 1:24 and 1:49 dilutions of from 0.8 to 1.0 per cent glycerol-methanol solutions of eosinates of methylene blue polychromed by the acid chromate process are fairly stable and often can be used for a second batch of slides after a first has been stained. Accordingly, successive lots of six slides were stained at 30-minute intervals in a single 50 c.c. lot of a 1:24 dilution of 1 per cent glycerol-methanol solution of polychrome methylene blue eosinate 44F. Of these, two slides were stained 10 minutes, two for 15 minutes, and two for 20 minutes. In all, seventy-eight slides were thus stained over a period of $7\frac{1}{2}$ hours. Of these, the best were the first two stained 20 minutes, but quite acceptable stains were obtained with 20-minute staining up to the fifth group. Longer staining probably would have given better results, but the 30-minute interval for starting a fresh lot did not permit this. This gradual loss of staining power is due both to exhaustion of stain and to spontaneous weakening of the solution by precipitation. A similar batch of stain used for staining after 7 hours' standing without being used gave better results than that in which seventy-two slides had been stained before, but the results were definitely inferior even to the seventh set stained in the 3-hour-old solution after thirty-six previous slides.

Following these experiences, attempts were made to apply the method of staining with a 1:24 dilution of a 1 per cent glycerol-methanol stock solution to commercial samples of Wright's stain. Stock solutions were thus prepared of lots AWr-2 and AWr-3. One set of rat trypanosomiasis blood films were stained 5, 7.5, 10, 15, 20, and 30 minutes in 1:24 dilutions, then another in 1:9 dilutions. Pale blue nuclei, failure to stain parasite chromatin, and slight and much precipitation, respectively, resulted in both trials. Parts of the 1 per cent stock solutions were then diluted with 4 volumes of methanol to 0.2 per cent, and other films were then stained on the slide in the usual way with 0.5 c.c. dilute stock stain to 1.5 c.c. buffered water. Good results were obtained.

In search of a possible explanation for this phenomenon, 10 c.c. of the glycerol-methanol stock solution was diluted with 88 c.c. distilled water and 2 c.c. glacial acetic acid and shaken with successive portions of carbon tetrachloride until no more red color was extracted. The CCl_4 was then shaken with two or three changes of saturated sodium carbonate solution, extracting from it a deep orange-red dye with yellow fluorescence on dilution and a spectroscopic absorption maximum in water at 516 $\text{m}\mu$, which agrees perfectly with eosin Y. The still red CCl_4 was then evaporated off on a water bath and the residue taken up in alcohol, to which it gave a deep violet color. This gave a spectral absorption peak at about 595 in alcohol. The acetic aqueous residue was blue violet in color and gave a single absorption maximum at 660 $\text{m}\mu$ (median of absorption band at 658).

The three fractions were regarded respectively as (1) eosin, (2) methylene violet, and (3) azures and methylene blue. It has been known since Bernthsen's time¹⁶ that on chloroform extraction of alkaline solutions of polychrome methy-

lene blue, azures and methylene violet pass into the chloroform, and when this in turn is shaken with dilute aqueous acetic acid, the azures pass into the weak acid while the methylene violet remains in the chloroform. Further, it is stated¹⁷ that with ether or chloroform extraction of an acetic acid solution, the azures remain in the aqueous solution, while the methylene violet passes into the chloroform or ether. The sodium carbonate wash was introduced purely to remove acetic acid from the fat solvent, and the appearance of a red dye in it was unexpected. On direct trial it was found that ether extraction of a 1 per cent acetic acid solution of eosin extracted a yellow coloring matter and that this ether solution imparted an orange-red color to aqueous sodium carbonate solution.

Recombination of the thus prepared azure methylene blue fraction with the eosin fraction, from the above Wright's stain, omitting the methylene violet fraction, gave satisfactory Romanovsky staining of blood films in a coplin jar when the dilution was of about the same depth of color as the 1:24 dilutions of the 1 per cent glycerol-methanol stock solution.

Since the foregoing indicated that methylene violet might be the substance responsible for the failure of certain samples to stain in coplin jar staining, it was determined to test this hypothesis further. To this end further trials of the fractionation method used above were made and chloroform substituted for ether or carbon tetrachloride, since it appeared to extract methylene violet much more rapidly. After a number of trials, the following method was evolved:

Dissolve 500 mg. Wright's stain in 25 c.c. methanol and 25 c.c. glycerol by shaking at frequent intervals for three days. Let settle over night. Pipette out 10 c.c. into a 120 c.c. bottle; add 88 c.c. distilled water and 2 c.c. glacial acetic acid. Mix thoroughly and let stand over night. Filter and shake filtrate in a separatory funnel with successive 50 c.c. portions of chloroform until last portion is pale pink or bluish. Combine chloroform extracts and shake with two successive 20 c.c. portions of 2 M sodium carbonate in a large separatory funnel. Combine the sodium carbonate fractions and neutralize cautiously with about 9 c.c. glacial acetic acid until further addition causes no further evolution of gas. Label and store this as "Eosin fraction of Wright's stain lot No. ---." Distill and recover the chloroform, using a water bath to heat the distilling flask. Take up the residue in successive 10 c.c. portions of absolute ethyl alcohol, filtering through filter paper to remove sodium salts, and make up final volume to 50 c.c. Label and store this as "Methylene violet fraction of Wright's stain lot No. ---." Store the aqueous acetic solution from which the chloroform extract was made in a 120 c.c. bottle, labeling "Azure fraction of Wright's stain, lot No. ---." Record total volume in cubic centimeters of each fraction, also dilution for spectrophotometry and spectroscopic data: λ (the absorption maximum), width of absorption band in which density is over 90 per cent of its maximum, the median of this band, and the optical density D at λ .

Since it appeared that the only adequate sample of commercial methylene violet on hand at the time contained a considerable proportion of azures, 5 Gm. of this was dissolved in some 1,200 to 1,500 c.c. of 2 per cent acetic acid and

extracted with chloroform, the chloroform extract was dried by distillation over a water bath, the acetic acid removed with a small amount of aqueous sodium carbonate solution, the again dry residue taken up in absolute alcohol, filtered, evaporated to dryness, removed as completely as possible, weighed to the nearest 0.1 mg., and dissolved in equal volumes of glycerol and methanol at 0.5 per cent concentration. The total yield was about 450 mg. This stock solution was diluted in appropriate amount and dilution for spectroscopic examination and the optical density determined and related to the amount of dry dye for comparison with the methylene violet fractions from the Wright's stain samples. This enabled the calculation in milligrams of the amount of methylene violet fraction recovered from the 10 c.c. of stock glycerol-methanol solution, which contained 100 mg. of Wright's stain.

Next, rat trypanosomiasis blood films were stained in a 1:24 dilution of each 1 per cent glycerol-methanol stock solution for 5, 7, 10, 15, 20, 30, 60, and 120 minutes in coplin jars, and on the slide in 2 parts of buffered water and 1 part of a dilute stock stain consisting of 2 c.c. of the 1 per cent glycerol-methanol solution and 8 c.c. methanol for 1, 2, 3, 5, 7.5, and 10 minutes. Water buffered to pH 6.5 was used throughout. All slides were examined under oil immersion and a numerical grade given in accordance with the following scheme:

- 7 Very dark cell nuclei, dark purple solid trophonuclei, heavy pink undulating membrane
- 6 Rather dark cell nuclei, solid trophonuclei, pink undulating membrane
- 5 Violet-purple cell nuclei, granular purple trophonuclei, clearly perceptible undulating membrane
- 4 Violet-purple cell nuclei, partially stained trophonuclei, well-stained blepharoplasts, indistinct undulating membranes
- 3 Rather light purple or red-purple nuclei, suggestion of trophonuclei, distinct blepharoplast
- 2 Blue-violet (-) → violet → violet-purple (+) cell nuclei, trophonuclei not shown, blepharoplasts, variable - to +
- 1 Blue nuclei deeper than cytoplasm, trophonuclei and blepharoplasts not shown
- 0 Unstained to pale blue or gray nuclei

Grade 7 is distinctly overstained; grades 5 and 6, good for trypanosomes; grades 4 and 5, good for blood work; grade 3, usable, grade 2, inferior; grades 1 and 0, useless.

The performance of two laboratory and twenty-six commercial samples of Wright's stain from four manufacturers, designated as A, B, D, and E, is given in Table I along with the assay figures for methylene violet recovered from a 100 mg. sample by the method given previously. Samples giving less than 4 mg. methylene violet gave satisfactory staining in coplin jars at 1:24 dilution of the 1 per cent glycerol-methanol solution. Some samples yielding around 4 mg. gave good staining after longer than the usual interval, while others did not. Samples with 5 mg. or more failed to give satisfactory stains in coplin jars. All but one sample gave satisfactory stains on the slide with a 1:2 dilution of an 0.2 per cent solution in 90 per cent methanol-10 per cent

glycerol (made by diluting the 1 per cent glycerol-methanol stock solution with 4 volumes of methanol).

Following these experiences it was decided to determine the effect of adding varying amounts of the purified methylene violet described above to the crude azure eosinate 44F. This last was made by acid chromate oxidation and should, according to Holmes,¹⁷ contain no methylene violet. The assay (Table I) indicated that only traces were present. In a previous similar trial a commercial methylene violet was used. It did not interfere with azure eosinate staining in jars and gave the gray-lilac cytoplasm and poor nuclear staining of azure C eosinate when higher proportions were used. A relatively large proportion of it remained in dilute acid aqueous solution when extracted by shaking with ether or chloroform. Consequently a methylene violet made by extracting with chloroform from a dilute acetic acid solution was used.

A series of stains was done in coplin jars with 2 c.c. 1 per cent glycerol-methanol solution of azure eosinate 44F in 48 c.c. of water buffered to pH 6.5, with the addition of graded amounts of an 0.5 per cent solution of repurified methylene violet in glycerol and methanol (equal volume mixture). The control series gave excellent stains, grade 6, on the grading schedule given above

TABLE I

WRIGHT'S STAIN PERFORMANCE IN COPLIN JAR AND "ON SLIDE" STAINING, CORRELATED WITH CRUDE METHYLENE VIOLET ASSAY

DYE	METHYLENE VIOLET MG. RECOVERED FROM 100 MG. DYE	COPLIN JAR		ON SLIDE		STAINING NOTES AND OTHER DILUTIONS
		1:24 DILUTION OF 1% GLYCEROL-METHANOL STOCK		1:2 DILUTION OF 0.2% IN 90% METHANOL-10% GLYCEROL		
		BEST GRADE	TIME MINUTES	BEST GRADE	TIME MINUTES	
A1		1	5 to 90	4 to 6	2 to 12	1:3 instead of 1:2
A2	5.71	1	5 to 90	5 to 6	2 to 12	1:3 instead of 1:2
B1	4.83	1	5 to 120	5	1 to 10	1:4 to 1:2 in 1 to 10 min.
B2	6.15	1	5 to 120	5	1 to 10	1:4 to 1 in 1 to 10 min.
B3	4.29	1	5 to 120	4 to 6	1 to 10	1:4 to 1:1 in 1 to 10 min.
B4	5.08	1	5 to 120	4 to 6	2 to 10	1:4 to 1 in 1 to 10 min.
B5		1	5 to 30	1	2 to 12	1:3 instead of 1:2
B6	0.60	5	15 to 30	4 to 6	2 to 12	1:3 instead of 1:2
C1	1.53	4 to 6	30 to 120	4 to 5	2 to 10	
D1	1.57	4 to 5	15 to 30	4 to 6	3 to 12	1:3 instead of 1:2
D2	1.51	5 to 6	7.5 to 120	4 to 5	2 to 10	
D3	2.64	4 to 5	7.5 to 120	5	1 to 10	
D4	1.99	5 to 6	7.5 to 120	4 to 5	1 to 10	
D5	1.01	4 to 5	15 to 30	4 to 6	2 to 12	1:3 instead of 1:2
D6	1.30	5 to 7	5 to 120	4 to 5	2 to 10	
D7	1.54	5 to 6	7.5 to 120	4 to 5	2 to 10	
D8	1.66	5 to 6	5 to 120	4 to 5	1 to 10	
E1	4.81	4	60 to 120	4 to 4	1 to 10	1:4 to 3:4 in 5 to 10 min.
E2	3.88	4 to 5	60 to 120	5 to 6	1 to 10	1:4 to 5 in 2 to 10 min.
E3	4.01	4 to 5	60 to 120	4 to 5	1 to 10	1:4 to 5 in 2 to 10 min.
E4	4.97	1	5 to 120	4 to 6	1 to 10	1:4 to 4:5 in 1 to 10 min.
E5	3.95	1 to 4	120	4 to 6	1 to 10	1:4 to 5 in 3 to 10 min.
E6	4.31	1	5 to 120	4 to 6	2 to 10	1:4 to 5 in 2 to 10 min.
E7	6.35	1	5 to 120	5	1 to 10	1:4 to 4 in 10 min.
E8	5.91	1	5 to 30	5 to 6	2 to 12	1:3 instead of 1:2
E9	4.31	1	5 to 120	4 to 5	1 to 10	1:4 to 5 in 3 to 10 min.
E10	3.85	1	5 to 120	4 to 5	2 to 10	1:4 to 3:6 in 1 to 10 min.
44F	0.06	4 to 6	7.5 to 30	5	2 to 12	1:3 instead of 1:2

in all periods from 15 minutes up to 2 hours. The series receiving 0.2 c.c. (1 mg.) (about 5 per cent of total dry stain) methylene violet gave a good stain, grade 5, in 2 hours, inferior stains in lesser intervals (grade 4 in 60 and 30 minutes, grade 2 in 20, and grade 1 in shorter intervals). With larger amounts of methylene violet (2, 3, 4, 5, 6, and 7 mg.) poor stains were regularly obtained.

This test was repeated, using 0.1, 0.15, 0.2, 0.25, 0.3, and 0.4 c.c. of the 0.5 per cent glycerol-methanol solution of methylene violet with 2 c.c. 1 per cent azure eosinate 44F and 48 c.c. water buffered to pH 6.5. In the control series, grade 5 or grade 6 stains were obtained in all periods from 15 to 120 minutes. With 0.1 c.c. (= 0.5 mg. or 2.5 per cent) methylene violet, similarly satisfactory stains were obtained in 30, 60, and 120 minutes but not in shorter times. With 0.15 c.c., the satisfactory stains, grade 5, were obtained only after 60 and 120 minutes. With 0.2 c.c. (= 1 mg.) a single satisfactory stain was obtained in 1 hour; those stained 30 and 120 minutes gave irregular, chiefly poor, results. With larger amounts of methylene violet no satisfactory stains were obtained.

A third similar test was made using azure eosinate 44F and methylene violet in such proportion that a total of 100 mg. dry dye per 100 c.c. glycerol-methanol stock solution was present. The first contained 100 mg. azure eosinate 44F alone; in the second this was reduced to 97.5 mg. and 2.5 mg. methylene violet added; and further mixtures contained 5, 7.5, and 10 mg. methylene violet with corresponding reduction of the azure eosinate. Of these stock mixtures there were made (A) a 1:24 dilution in water buffered to pH 6.5; (B) a 1:24 dilution containing 8 per cent acetone in the buffered water; and (C) a 1:49 dilution with 8 per cent acetone in buffered water.

The three controls without methylene violet gave (grades 5 and 6) excellent stains in from 10 to 120 minutes. With 2.5 per cent methylene violet, the acetone mixtures gave excellent stains in from 10 to 120 minutes; that without acetone, in from 15 to 120 minutes. With 5 per cent methylene violet all three mixtures stained moderately well in from 30 to 120 minutes, and with 7.5 per cent and 10 per cent methylene violet satisfactory stains were not obtained up to 120 minutes with any of the mixtures.

Since the foregoing trials indicated that this methylene violet seriously interfered with staining in coplin jars, it was decided to discover whether it had any deleterious effect in "on slide" staining in the traditional manner. Accordingly, a series of dilutions of the foregoing stock mixtures in methanol was made, bringing the dye concentration down from 1 per cent to 0.2 per cent (200 mg. per 100 c.c.). Three series were stained for 1, 2, 3, 5, 7.5, and 10 minutes. The first was diluted 4 c.c. stain to 8 c.c. water; the second, 3 c.c. stain to 9 c.c. water; the third, 2.5 c.c. stain to 10 c.c. water. All the water was buffered to pH 6.5. Each series comprised mixtures of azure eosinate with 0, 2.5, 5, 7.5, 10, 12.5, 15, 20, and 25 per cent methylene violet.

The first dilution series, 1 stain plus 2 water, gave good stains in all periods from 2 to 10 minutes, regardless of the amount of methylene violet. The second series, 1 stain plus 3 water, gave good stains in from 2 to 10 minutes with methylene violet levels up to 12.5 per cent, varying to poor with

higher levels. In the third series, 1 stain plus 4 water, good results were obtained in the controls and with 2.5, 5, and 7.5 per cent methylene violet, variable with 10, 12.5, and 15 per cent, and poor with 20 and 25 per cent. None of the stains were fully satisfactory with 1-minute staining, but with 2 minutes or more, longer periods gave little increase in density of staining. All films were fixed in methanol prior to staining, and all stain-water mixtures were made in test tubes and at once pipetted onto the slides. Uniform mixtures were thereby assured.

As a further control, this last experiment was repeated, using a freshly obtained commercial sample of methylene violet (NLv-4) at 0, 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 15, 20, 25, and 30 per cent levels. With 1:24 dilution of the glycerol-methanol stock solution, no levels above 10 per cent were tested, since fully satisfactory staining was obtained only with 4 per cent or less methylene violet. Good staining in prolonged intervals was obtained in from 5 to 7 per cent, and in 8 and 10 per cent satisfactory staining was not attained in 4 hours.

With the stock solutions diluted to 0.2 per cent with methanol, dilutions of 1:4, 1:3, 1:2, and 5:7 were employed and the 5 and 7 per cent methylene violet levels omitted. All slides were studied at the 0, 3, 6, 10, 15, and 30 per cent levels; in addition, all the 1:4 and 5:7 dilution slides were also studied at the 20 and 25 per cent levels and the 1:4 dilution slides at the 12 per cent level. Three- and 5-minute stains were made of all the 1:2 dilution slides.

The 1:4 dilution gave good stains up to 10 per cent methylene violet, fair and variable at from 12 to 25 per cent, and poor at 30 per cent. The 1:3 and 1:2 dilutions gave good stains at all methylene violet levels, and with the 5:7 dilution, higher methylene violet levels actually gave somewhat better staining than lower concentrations, particularly with shorter staining intervals. The same difference was seen to a less degree with the 1:2 dilution. With the unmodified (control) azure eosinate, 1:4, 1:3, and 1:2 dilutions required 3 minutes for satisfactory stains, while 5 minutes were needed with the 5:7 dilution. With higher methylene violet concentration, 3- and sometimes 2-minute stains were satisfactory with the 1:3, 1:2, and 5:7 dilutions.

The dilutions used in the two last cited experiments lie within the range of those attained by the drop-for-drop dilution technique commonly used. Wright⁶ originally directed placing some of the methanol solution on the preparation to fix it for 1 minute, and then added distilled water until a metallic sheen formed. Later¹⁸ he directed adding a noted quantity of stain from a medicine dropper and a minute later the same quantity of water also from a medicine dropper; in 1924¹⁹ he directed the use of 10 drops of the stain and 10 drops of water. Mal-lory²⁰ later returned to the use of equal quantities from a pipette, while Conn¹⁴ copied the 1924 directions of Wright. Many workers, however, do not realize that an equal number of drops is not an equal quantity when a methanol solution is compared with water.

The number of drops per cubic centimeter and average drop size in cubic millimeters for a 0.3 per cent methanol solution of azure eosinate, for a 1.0 per cent glycerol-methanol solution of the same azure eosinate, and for distilled water is listed in Table II. Ten serologic pipettes were used, five with blunt and five with sharp points; five bulb pipettes were used in two positions, nearly

TABLE II
NUMBER OF DROPS PER CUBIC CENTIMETER AND DROP SIZES FOR VARIOUS DROPPERS
SOLUTIONS OF AZURE EOSINATES

TYPE	NO.	0.3% METH.		1.0% GLYCEROL METHANOL		DISTILLED WATER	
		TOTAL DROPS AND C.C. COUNTED	ER S C.C.	TOTAL DROPS AND C.C. COUNTED	NUMBER DROPS PER 1 C.C.	TOTAL DROPS AND C.C. COUNTED	NUMBER DROPS PER 1 C.C.
Blunt serologic 1 c.c. pipette	1	175 = 4 c.c.		158 = 4 c.c.	39.5	48 = 3 c.c.	16.0
	2	181 = 4 c.c.		156 = 4 c.c.	39.0	48 = 3 c.c.	16.0
	3	179 = 4 c.c.		158 = 4 c.c.	39.5	48 = 3 c.c.	16.0
	4	178 = 4 c.c.		157 = 4 c.c.	39.2	49 = 3 c.c.	16.3
	5	134 = 3 c.c.		155 = 4 c.c.	38.7	48 = 3 c.c.	16.0
Sharp serologic 1 c.c. pipette	6	169 = 3 c.c.		1253 = 3 c.c.	49.0	66 = 3 c.c.	22.0
	7	177 = 3 c.c.		1190 = 3 c.c.	49.3	67 = 3 c.c.	22.3
	8	179 = 3 c.c.		1208 = 3 c.c.	50.7	69 = 3 c.c.	23.0
	9	173 = 3 c.c.		1065 = 3 c.c.	47.7	66 = 3 c.c.	22.0
	10	171 = 3 c.c.		1173 = 3 c.c.	48.0	66 = 3 c.c.	22.0
Bulb pipette held nearly vertical	11	1429 = 25 c.c.		1105 = 25 c.c.	19.9	577 = 25 c.c.	23.1
	12	1284 = 25 c.c.		1067 = 25 c.c.	47.6	524 = 25 c.c.	21.1
	13	1273 = 25 c.c.		957 = 25 c.c.	48.7	580 = 25 c.c.	23.5
	14	1100 = 25 c.c.	44.0	1065 = 25 c.c.	42.6	464 = 25 c.c.	18.6
	15	1203 = 25 c.c.	48.2	1173 = 25 c.c.	46.9	495 = 25 c.c.	19.8
Bulb pipette held nearly horizontal	16	1199 = 25 c.c.	48.0	1105 = 25 c.c.	44.3	580 = 25 c.c.	23.2
	17	902 = 25 c.c.	38.5	1067 = 25 c.c.	42.7	460 = 25 c.c.	18.4
	18	1010 = 25 c.c.	40.4	957 = 25 c.c.	38.4	501 = 25 c.c.	20.0
	19	935 = 25 c.c.	37.4	999 = 25 c.c.	40.0	405 = 25 c.c.	16.2
	20	961 = 25 c.c.	38.4	1135 = 25 c.c.	45.4	467 = 25 c.c.	18.7
Flat- topped dropping bottles	21	1449 = 40 c.c.	36.3	978 = 30 c.c.	32.6	414 = 30 c.c.	13.8
	22	1089 = 30 c.c.	36.3	1012 = 30 c.c.	33.7	407 = 30 c.c.	14.3
	23	1010 = 30 c.c.	33.5	973 = 30 c.c.	32.4	429 = 30 c.c.	14.3
	24	1042 = 30 c.c.	34.7	982 = 30 c.c.	32.7	423 = 30 c.c.	14.1
	25	1045 = 30 c.c.	34.8	980 = 30 c.c.	32.7	420 = 30 c.c.	14.0
Rabbit- can- topped dropping bottles	26	962 = 25 c.c.	38.5	918 = 25 c.c.	36.7	381 = 25 c.c.	15.2
	27	976 = 25 c.c.	39.0	898 = 25 c.c.	35.9	402 = 25 c.c.	16.1
	28	950 = 25 c.c.	38.0	886 = 25 c.c.	35.5	371 = 25 c.c.	14.8
	29	930 = 25 c.c.	38.0	930 = 25 c.c.	37.2	341 = 25 c.c.	13.6
	30	1068 = 25 c.c.	42.7	1017 = 25 c.c.	40.7	185 = 25 c.c.	7.4
30		1023 = 25 c.c.	40.9	918 = 25 c.c.	36.7	347 = 25 c.c.	13.9
30						202 = 25 c.c.	8.1
30						175 = 25 c.c.	7.0
30						297 = 25 c.c.	9.1
30						305 = 25 c.c.	12.2
30							82.0

vertical and nearly horizontal, and ten dropping bottles of two different designs, five each, were tried. With the last of these, No. 30, six consecutive trials were made with water, two each with the two stain solutions.

Taking the extremes in drop sizes, a drop-for-drop dilution of the methanol solution could vary from 41 per cent stain or 1:1.44 to 10.5 per cent or 1:8.5. If bottle 30, which gave extraordinarily large drops of water, were neglected in the calculation, the lower dilution would be 18.6 per cent or 1:4.39. This last dilution approaches a level where stains with higher than 7.5 per cent methylene violet do poorly in "on slide" staining.

It may be noted further that the traditional 1 drop per 1 c.c. dilution for Giemsa stain would range, on the basis of the azure eosinate solution in equal volumes of glycerol and methanol, from 1:33.4 to 1:50.8. This variation is enough to make a very considerable difference in the density of staining obtainable in from 30 to 45 minutes, particularly with reference to such structures as Schüffner's granules and undulating membranes.

Since some authors have recommended equal volumes of Wright's stain and water for staining (though I am sure they meant an equal number of drops), it was decided to determine the effect of such high concentration of methanol solution. Some years ago we tried 1:1 dilutions and abandoned them for a 1:2 dilution because of inferior performance.

A 0.3 per cent methanol solution of azure eosinate 44D was used. Mixtures with buffered water (pH 6.5) were made in test tubes containing, respectively, 60, 55, 50, 45, 40, 35, and 33 per cent by volume of the stock stain, and 1.5 c.c. quantities at once deposited on previously fixed rat trypanosomiasis blood films. These were stained 1, 2, 3, 5, 7, 5, and 10 minutes. Generally, all the 1-minute films were inferior (grade 2 on the grading schedule). The 33 per cent dilution gave good stains in periods of 2 minutes or more, with fair to good differentiation of trypanosome nuclei in periods of from 5 to 10 minutes. At 45 per cent dilution, trypanosome trophonuclei were poorly stained in from 7.5 to 10 minutes, with fairly good blood staining. With 50 to 60 per cent stain, trophonuclei were not stained, and leucocyte staining was only fair. Really satisfactory blue lymphocyte and trypanosome cytoplasm was not attained at any concentration higher than 33 per cent. With increasing concentration, cytoplasm altered in color through lilac to pinkish gray.

SUMMARY

The constitution of the azure component of Wright's stain is difficult to control when the traditional process of steaming one hour with NaHCO_3 is used. This difficulty is largely overcome in commercial manufacture by spectrographic control.

Substitution of the acid chromate oxidation process gives relatively constant azure constitution when the same proportions of methylene blue and potassium (or sodium) bichromate are used.

Preparation of thiazin eosinates containing chiefly azure B and methylene blue is suggested on account of the greater stability of these in methanol solution than azure A eosinates.

Satisfactory azure eosinates are readily prepared from the zinc chloride methylene blue. In calculation of molar equivalents, the presence of 19.5 per cent of water and zinc chloride must be allowed for.

There are distinctly different limitations on staining in covered jars and on open slides. In covered jars methanol percentages of 15 per cent or more are distinctly inhibitory to azure staining of nuclei, while on open slides 33 per cent methanol is tolerated. Here, too, higher than 35 per cent methanol definitely impairs staining of leucocytes and especially parasites. When evaporation of methanol is retarded in "on slide" staining, the usual 25 to 33 per cent dilutions fail to stain them.

Distinctly sharper staining of chromatin and clearer cytoplasmic colors are obtained by longer staining in more dilute solutions, even when the same thiazin eosinate is employed.

The solubility of a mixed methylene blue azure B eosinate in equal volumes of methanol and glycerol is in the neighborhood of 1.1 per cent or 11 mg. per cubic centimeter.

Quite satisfactory staining may be attained in from 15 to 20 minutes with a 1:24 dilution of a 1 per cent glycerol-methanol solution of such an eosinate in covered jar staining. Such solutions may be re-used but with somewhat inferior results.

Many samples of commercial Wright stain do not permit staining by this technique; others perform beautifully. It was found that the first group of samples contained higher proportions of methylene violet than the second. In confirmation of this finding it was shown that chemical fractionation of a Wright stain of the first group into azure, eosin, and methylene violet fractions and recombination of the first two fractions would give successful staining from dilute aqueous solution. It was further shown that addition of purified methylene violet to a 1 per cent glycerol-methanol solution of an azure eosinate in 2.5 per cent level retarded satisfactory staining in the 1:24 aqueous dilution, with 5 per cent retardation was greater, and with 7.5 per cent satisfactory staining was inhibited. With a commercial methylene violet 4 per cent was tolerated, from 5 to 7 per cent retarded staining, and from 8 to 10 per cent inhibited.

However, when a 200 mg. per 100 c.c. methanol solution of the same azure eosinate was used with 1:2, 1:3, and 1:4 dilutions in "on slide" staining, the 1:2 dilution gave satisfactory stains with substitution of as much as 25 per cent methylene violet, the 1:3 dilution tolerated methylene violet substitution up to 12.5 per cent of the total dry dye, and the 1:4 dilution up to 7.5 per cent only. Higher levels of a commercial methylene violet were tolerated and appeared to improve staining with higher initial methanol concentration in the final stain mixture.

It would appear that methylene violet is kept in solution by the presence of methanol rather than by the azures, as previously assumed, and that when insufficient methanol (or other solvent) is present to keep the methylene violet in solution, it precipitates and carries the azure eosinates down with it.

Drop-for-drop dilutions of methanol solutions of azure eosinates with water do not constitute equal volume mixtures but vary from about 1:1.5 to 1:4.5 (41 per cent to 19 per cent).

One drop to 1 c.c. dilutions of glycerol-methanol solutions of azure eosinate vary from about 1:35 to 1:52 in volume dilution.

CONCLUSIONS

The presence of any considerable amount, over 4 per cent, of methylene violet is tolerated in Romanovsky staining only in "on slide" staining with relatively high initial concentration of methanol in the final staining mixture. With concentrations below 20 per cent, such as are reached in Giemsa type methods, not much over 4 to 5 per cent methylene violet is tolerated. Larger amounts occasion precipitation of both the methylene violet and the thiazin eosinates. However, in "on slide" staining with methanol concentrations of from 30 to 40 per cent, the presence of from 15 to 30 per cent methylene violet distinctly improves azure eosinate staining. For all concentrations of methylene violet, from 25 to 33 per cent initial methanol concentration appears to be the best range for "on slide" staining. Higher concentrations give poorer staining with low methylene violet content in the dye; lower concentrations occasion precipitation with higher methylene violet levels. The latter limitation is the less important as the usual Wright stains appear to contain less than 7.5 per cent methylene violet, and this amount is tolerated even by a 20 per cent dilution of the methanol stock solution.

Alkali polychroming methods are difficult to control so as to produce constant proportions of the various azures, and they also produce a variable amount of methylene violet. Acid chromate polychroming is easily controlled, yields relatively constant products, and produces little or no methylene violet. It permits the use of the commercial double zinc chloride as well as of medicinal methylene blue.

Acid chromate polychroming with 200 mg. $K_2Cr_2O_7$ (or its Na equivalent) per gram of 86 per cent medicinal methylene blue (20 per cent less $K_2Cr_2O_7$ for the zinc salt) yields chiefly azure B with some methylene blue. This gives good staining of blood protozoa as well as of blood cells. Its eosinate is relatively more stable in methanol solution than eosinates containing higher proportions of azure A. The use of a 1:24 dilution of a 1 per cent solution of such an eosinate in equal volumes of glycerol and methanol can be highly recommended for blood staining. Further, this glycerol-methanol solution is much more stable than plain methanol solutions and can be used as a stock solution for dilution with methanol to 150 or 200 mg. per 100 c.c. for the usual "on slide" Wright staining technique.

Acknowledgment is due Medical Technician Daisy Plotka and Junior Chemist Dorothy C. Peterson for aid in staining and spectroscopic procedures.

REFERENCES

1. Roe, M. A., Lillie, R. D., and Wilcox, A.: American Azures in the Preparation of Satisfactory Giemsa Stains for Malaria Parasites, *Pub. Health Rep.* 55: 1272-1278, 1940.
2. Lillie, R. D., and Roe, M. A.: Studies on Polychrome Methylene Blue. I. Eosinates, Their Spectra and Staining Capacity, *Stain Technol.* 17: 57-63, 1942.
3. Lillie, R. D.: Studies on Polychrome Methylene Blue. III. Alkali Methods of Polychroming, *Stain Technol.* 18: 1-11, 1943.
4. Lillie, R. D.: Blood and Malaria Parasite Staining with Eosin Azure Methylene Blue Methods, *Am. J. Pub. Health* 33: 948-951, 1943.

5. Lillie, R. D.: Deterioration of Romanovsky Stain Solutions in Various Organic Solvents, Pub. Health Rep. (suppl.). In press.
6. Wright, J. H.: A Rapid Method for the Differential Staining of Blood Films and Malarial Parasites, J. Med. Research 7: 134-144, 1902.
7. MacNeal, W. J.: Methylene Violet and Methylene Azure, J. Infect. Dis. 3: 412-33, 1906.
8. MacNeal, W. J.: Methylene Violet and Methylene Azure A and B, J. Infect. Dis. 36: 538-46, 1925.
9. Holmes, W. C., and French, R. W.: The Oxidation Products of Methylene Blue, Stain Technol. 1: 17-26, 1926.
10. Lillie, R. D.: Studies on Polychrome Methylene Blue. II. Acid Oxidation Methods of Polychroming, Stain Technol. 17: 97-110, 1942.
11. Lillie, R. D.: A Giemsa Stain of Quite Constant Composition and Performance Made in the Laboratory From Eosin and Methylene Blue, Pub. Health Rep. 58: 449-452, 1943.
12. Lillie, R. D.: Spectroscopic Data on Paired mixtures of Purified Thiazins and on Commercial Thiazins, Pub. Health Rep. (suppl.). In press.
13. Mallory, F. B., and Wright, J. W.: Pathological Technique, ed. 4, Philadelphia, 1908, W. B. Saunders Co., pp. 365-70.
14. Conn, H. J.: Biological Stains, ed. 4, Geneva, N. Y., 1940, Biotech. Publ., p. 173.
15. Rowe, F. M.: Colour Index, Bradford, Yorkshire, 1924, Society of Dyers and Colourists.
16. Bernthsen, A.: Studien in der Methylenblaugruppe, Liebigs Ann. d. Chem. 230: 73, 1885.
17. Holmes, W. C.: Subsidiary Dyes in Methylene Blue, Stain Technol. 2: 71-73, 1927.
18. Mallory, F. B., and Wright, J. H.: Pathological Technique, ed. 6, Philadelphia, 1918, W. B. Saunders Co., pp. 382-385.
19. Mallory, F. B., and Wright, J. H.: Pathological Technique, ed. 8, Philadelphia, 1924, W. B. Saunders Co., pp. 471-72.
20. Mallory, F. B.: Pathological Technique, Philadelphia, 1938, W. B. Saunders Co., pp. 181-183.

SEROFLOCCULATION WITH A NEW GROUP OF ANTIGENS IN SOME PATHOLOGIC CONDITIONS

"PATHOGNOSTIC SEROFLOCCULATION"

L. ROSENTHAL, M.D.
BROOKLYN, N. Y.

POSITIVE reactions with serodiagnostic tests for syphilis have been reported occasionally in diseases other than syphilis. Among the conditions which give such so-called false positive reactions, Stokes¹ mentions frambesia tropica, recurrent fever, trypanosomiasis, spotted fever, leprosy, scarlet fever, ulcus tropicum, endocarditis, septicemia, malaria, ulcus molle, lupus erythematosus, leukemia, pernicious anemia, mycosis fungoides, tuberculosis, anesthesia, malignant tumors, psoriasis, pemphigus, aphthae, Graves' disease, plumbism, diabetes, eclampsia, leishmaniasis, pellagra, beri-beri, and pregnancy.

With the refinement of the technique of the tests and with the improvements introduced in the preparation of the beef heart antigens, the number of conditions which produce so-called false positive reactions have decreased considerably. A list compiled by Eagle² in 1937 contained only six such diseases: trypanosomiasis, relapsing fever, infectious mononucleosis, malaria, leprosy, and yaws. Recently, however, with the widespread routine performance of serodiagnostic tests for syphilis, attention was again drawn to the occurrence of positive reactions in the absence of syphilitic infection. Eagle,³ in 40,545 white students of both sexes in 25 universities, found 36 false positive reactions. Other workers (Moore, Eagle, Mohr⁴) reported false positive reactions in acute infections of the respiratory tract including pneumonia, and after smallpox vaccination (Lynch et al.⁵). The problem of the specificity of the syphilitic serodiagnostic tests was further complicated by the discovery that sera of normal animals give positive reactions (Porro,⁶ Greene et al.⁷).

According to Kahn,⁸ the true positive reactions can be distinguished from false or nonspecific reactions of human and animal sera by a special verification test in which different temperatures and salt concentrations for each type of reaction are used. True positive reactions are stronger at 37° C. and weaker at 1° C., while nonspecific reactions are weaker at 37° C. and stronger at 1° C. Specific serologic reactions tend to become stronger, and nonspecific weaker as the salt concentration of the reacting system is increased. Kahn⁹ was able to obtain a very high percentage (95-98 per cent) of positive nonspecific reactions in Kahn negative sera by performing the test at low temperature, with unheated sera and with the use of a special sensitive antigen prepared from beef heart powder which was kept at 37° for several months. Based on these facts, Kahn recognizes the existence of a universal biologic type of reaction which can be dis-

From the Department of Laboratories, Israel Zion Hospital.
Received for publication, July 22, 1944.

covered under proper conditions, which is non-specific in nature and has no connection with syphilitic infection.

It is generally assumed that syphilitic sera contain a substance designated as reagin which produces flocculation in colloidal suspensions of beef heart extracts. Many investigators (Barnett et al.,¹⁰ Sherwood et al.,¹¹ Lund¹²) believe that the so-called false positive reactions are due also to the presence of small amounts of reagin-like substances in nonsyphilitic sera. The opinion has, therefore, been expressed that the difference between true and false positive reactions is of a quantitative rather than a qualitative nature (Kolmer¹³).

From this short survey, it follows that up to the present the investigations of serodiagnostic tests were mainly concerned with syphilis. All the other pathologic conditions giving false positive reactions were regarded as side issues and received attention only insofar as they interfered with the differential diagnosis of syphilis. In any case, the low incidence of false positive reactions in each pathologic group permitted no definite conclusions as to their significance. Nevertheless, the fact remains that some pathologic non-syphilitic sera have a tendency to give positive flocculation tests.

It seemed worth while to detach the problem of seroflocculation from syphilis and explore the possibility of finding antigens which will bring out the tendency of seroflocculation in various other pathologic conditions in a constant and distinct way. It was clear that cholestermized antigens prepared from beef heart would not serve this purpose since the standard antigens rarely gave positive reactions outside of syphilis, while the antigens prepared by Kahn from aged beef heart powder approached universal sensitivity by giving flocculation with nearly all sera, normal and pathologic alike.

In quest for a suitable antigen, we first tested alcoholic extracts prepared from various dry substances containing lipid materials. They were milk powder, egg yolk powder, soya bean flour, jack bean meal, wheat flour, pulverized walnuts, almonds and dried yeasts. These extracts fortified by cholesterol gave positive flocculation reactions in some pathologic conditions but in a casual and non-uniform way, and therefore were discarded. In continuing our search for new sources, we finally selected the following substances for the preparation of antigens: I. *Animal Origin*—butter, chicken fat, human fat, lanolin (anhydrous), oil of lard, neat's-foot oil, sperm oil, suet; II. *Plant Origin*—oil of sweet almonds, castor oil, cocoanut oil, cottonseed oil, hydrogenated plant oil (Crisco), linseed oil, olive oil, peanut oil, poppy seed oil, sesame oil, soya bean oil, oil of walnut; III. *Mineral Oil* (*Petrolatum liquidum*).

TECHNIQUE

A. *Preparation of the Antigen*.—Stock solution: 1 ml. of oil or fat (when necessary after melting) was added to 100 ml. of absolute alcohol and the mixture was vigorously shaken. If some turbidity appeared, the mixture was placed in a water bath at 56° C. for 15 minutes and cooled at room temperature. When a sediment formed, it was removed by filtration. By addition of absolute alcohol to the stock solution, concentrations of less than 1 per cent could be prepared. The optimal concentration of the stock solution to be used in the test was determined by titration (see under D). We prepared alcoholic solutions from all the various substances listed above and tested each of them. Since, generally,

sera containing reagin often fail to react with the new antigens, it may be assumed that the factor producing pathognostic seroflocculation is of a nature other than reagin. Furthermore, due to differences in the results of the blood sedimentation rate and the seroflocculation test, there is apparently no common factor responsible for both tests. In this first stage of our investigation, all our efforts were devoted to devising the test, selecting the antigens, perfecting the details of the technic and studying the general aspects of the phenomenon of "pathognostic seroflocculation." So far, no attempt was made to correlate the results of the test with the clinical findings, the duration of the disease, severity and extent of the process, complications or type of treatment. At present, it can be stated that a positive test suggests the presence of infection, inflammation or malignancy. It remains for further studies to investigate the various problems connected with the test and to find out its practical value for diagnostic or prognostic purposes.

SUMMARY

New antigens were prepared from paraffin oil and from fats and oils of plant and animal origin. The technique of testing sera with these antigens is described. The test gives negative results with sera of healthy persons and positive results in a majority of cases of acute infections, especially pneumonia, and in tuberculosis, cancer and coronary thrombosis. Four plus Wassermann and Kahn positive sera often fail to react with these antigens. The differences between this type of seroflocculation and the biologic and syphilitic type are discussed. The name "pathognostic seroflocculation" is proposed for the test.

REFERENCES

1. Stokes, J. H.: *Modern Clinical Syphilology*, Philadelphia, W. B. Saunders Co., 1934, p. 131.
2. Eagle, H.: *The Laboratory Diagnosis of Syphilis*, St. Louis, The C. V. Mosby Company, 1937, p. 317.
3. Eagle, H.: On Specificity of Serologic Tests for Syphilis as Determined by 40,545 Tests in College—Student Population, *Am. J. Syph., Gonorr. and Ven. Dis.* 25: 7, 1941.
4. Moore, I. E., Eagle, H., Mohr, Ch. F.: Biologic False Positive Serologic Tests for Syphilis; Suggested Method of Approach to Their Clinical Study, *J. A. M. A.* 115: 1602, 1940.
5. Lynch, F. W., Boynton, R. E., Kimball, A. C.: False Positive Serologic Reactions for Syphilis Due to Smallpox Vaccinations (Vaccinia), *J. A. M. A.* 117: 591, 1941.
6. Porro, T. J.: The Kahn Reaction With Serum of Different Animals, *J. Infect. Dis.* 53: 210, 1933.
7. Greene, R. A., Harding, H. B., Hudspeth, W. T., Pistor, W. J.: Reaction of Sera of Different Animals to Kahn, Kline, Ide, Eagle, and Laughlin Tests, *J. LAB. & CLIN. MED.* 23: 763, 1938.
8. Kahn, R. L.: Verification Test in Serology of Syphilis, *J. LAB. AND CLIN. MED.* 28: 1175, 1943.
9. Kahn, R. L., Marcus, S., McDermott, E. B., and Adler, I.: Serologic (Non-Syphilitic) Reaction Approaching Universal Sensitivity, *J. Invest. Dermat.* 5: 459, 1942.
10. Barnett, C. W., Jones, R. B., and Kulchar, G. W.: Measurement of Reagin in Non-syphilitic Sera, *Proc. Soc. Exper. Biol. & Med.* 33: 214, 1935.
11. Sherwood, N. P., Bond, G. C., Canuteson, R. I.: On Possible Presence of Reagin-like Factor in Normal Human Serum, *Am. J. Syph., Gonorr. and Ven. Dis.* 25: 179, 1941.
12. Lund, H.: Titration of Traees of Reagin: Technique of Flocculation Using Maximal Serum Proportions With Secondary Recovery of Antigen, *Am. J. Syph., Gonorr. and Ven. Dis.* 26: 1, 1942.
13. Kolmer, J. A.: The Problem of Falsely Doubtful and Positive Reactions in the Serology of Syphilis, *Am. J. Pub. Health* 34: 510, 1944.

"REVERSE" TYPING AS AN AID IN THE DIAGNOSIS OF PNEUMONIA

E. RACKER, M.D.,* AND S. P. ROSE, M.D.
NEW YORK, N. Y.

THE clinical similarity between atypical pneumococcal pneumonia and sporadic cases of primary atypical pneumonia makes a differentiation very difficult.¹ Pneumococcal pneumonia may often fail to show the invasive organism in the sputum. Several mechanisms could be responsible for this. If the pneumonic lesions are small and scattered, they may not communicate with a larger bronchus, similar to miliary tuberculosis of the lung; the communicating bronchus may be blocked by a mucous plug; the cough reflex may be suppressed in some patients; and finally, the sputum may contain inhibitory factors or bacteriolysins which may prevent satisfactory typing of the organisms. It is also well known that in children with pneumonia a satisfactory sputum specimen is frequently unobtainable. In these cases a method which would not depend on the bacteriologic sputum studies would be of diagnostic value.

About 70 per cent of patients with pneumonia develop specific antibodies between the seventh and tenth day of illness as determined by the passive protection of mice.² A simple method has therefore been adopted to utilize these antibodies diagnostically.

Method.—Fifty-six strains of pneumococci were prepared as follows: The virulent strains were passed through mice and grown in a 1 per cent baeto-peptone broth containing from 5 to 10 per cent horse serum. After eighteen hours' growth, the cultures were centrifuged and the sedimented bacteria washed with saline. Saline suspensions of the recentrifuged organisms were then incubated in a water bath at 56° C. for one hour. Final dilutions containing 1 per cent formalin and about 0.2 mg. bacteria (dry weight) per cubic centimeter were made. The pneumococci of these suspensions showed distinct capsule swelling when mixed with a drop of specific antiserum containing from 5 to 10 units antibody per cubic centimeter. The strains were pooled in groups of six to simplify the diagnostic procedure.

Using the patient's serum as antibody, the pooled strains were examined for quellung reaction as in the standard Neufeld method. If a patient's serum gave negative results at the onset of disease, and specific capsule-swelling antibodies in rising titers appeared later, the test was considered positive.

Agglutinin titers were determined on all specimens of serum which were typed by this method. In all instances the results of the agglutination reaction confirmed those obtained by the Neufeld reaction.

*Present address: Department of Bacteriology, New York University College of Medicine.
From the Pneumonia Service, Harlem Hospital.
Received for publication, May 3, 1944.

"REVERSE" TYPING AS AN AID IN THE DIAGNOSIS OF PNEUMONIA

E. RACKER, M.D.,* AND S. P. ROSE, M.D.
NEW YORK, N. Y.

THE clinical similarity between atypical pneumococcal pneumonia and sporadic cases of primary atypical pneumonia makes a differentiation very difficult.¹ Pneumococcal pneumonia may often fail to show the invasive organism in the sputum. Several mechanisms could be responsible for this. If the pneumonic lesions are small and scattered, they may not communicate with a larger bronchus, similar to military tuberculosis of the lung; the communicating bronchus may be blocked by a mucous plug; the cough reflex may be suppressed in some patients; and finally, the sputum may contain inhibitory factors or bacteriolysins which may prevent satisfactory typing of the organisms. It is also well known that in children with pneumonia a satisfactory sputum specimen is frequently unobtainable. In these cases a method which would not depend on the bacteriologic sputum studies would be of diagnostic value.

About 70 per cent of patients with pneumonia develop specific antibodies between the seventh and tenth day of illness as determined by the passive protection of mice.² A simple method has therefore been adopted to utilize these antibodies diagnostically.

Method.—Fifty-six strains of pneumococci were prepared as follows: The virulent strains were passed through mice and grown in a 1 per cent bacto-peptone broth containing from 5 to 10 per cent horse serum. After eighteen hours' growth, the cultures were centrifuged and the sedimented bacteria washed with saline. Saline suspensions of the recentrifuged organisms were then incubated in a water bath at 56° C. for one hour. Final dilutions containing 1 per cent formalin and about 0.2 mg. bacteria (dry weight) per cubic centimeter were made. The pneumococci of these suspensions showed distinct capsule swelling when mixed with a drop of specific antiserum containing from 5 to 10 units antibody per cubic centimeter. The strains were pooled in groups of six to simplify the diagnostic procedure.

Using the patient's serum as antibody, the pooled strains were examined for quellung reaction as in the standard Neufeld method. If a patient's serum gave negative results at the onset of disease, and specific capsule-swelling antibodies in rising titers appeared later, the test was considered positive.

Agglutinin titers were determined on all specimens of serum which were typed by this method. In all instances the results of the agglutination reaction confirmed those obtained by the Neufeld reaction.

*Present address: Department of Bacteriology, New York University College of Medicine.
From the Pneumonia Service, Harlem Hospital.
Received for publication, May 3, 1944.

sera containing reagin often fail to react with the new antigens, it may be assumed that the factor producing pathognostic seroflocculation is of a nature other than reagin. Furthermore, due to differences in the results of the blood sedimentation rate and the seroflocculation test, there is apparently no common factor responsible for both tests. In this first stage of our investigation, all our efforts were devoted to devising the test, selecting the antigens, perfecting the details of the technic and studying the general aspects of the phenomenon of "pathognostic seroflocculation." So far, no attempt was made to correlate the results of the test with the clinical findings, the duration of the disease, severity and extent of the process, complications or type of treatment. At present, it can be stated that a positive test suggests the presence of infection, inflammation or malignancy. It remains for further studies to investigate the various problems connected with the test and to find out its practical value for diagnostic or prognostic purposes.

SUMMARY

New antigens were prepared from paraffin oil and from fats and oils of plant and animal origin. The technique of testing sera with these antigens is described. The test gives negative results with sera of healthy persons and positive results in a majority of cases of acute infections, especially pneumonia, and in tuberculosis, cancer and coronary thrombosis. Four plus Wassermann and Kahn positive sera often fail to react with these antigens. The differences between this type of seroflocculation and the biologic and syphilitic type are discussed. The name "pathognostic seroflocculation" is proposed for the test.

REFERENCES

1. Stokes, J. H.: *Modern Clinical Syphilology*, Philadelphia, W. B. Saunders Co., 1934, p. 131.
2. Eagle, H.: *The Laboratory Diagnosis of Syphilis*, St. Louis, The C. V. Mosby Company, 1937, p. 317.
3. Eagle, H.: On Specificity of Serologic Tests for Syphilis as Determined by 40,545 Tests in College—Student Population, *Am. J. Syph., Gonorr. and Ven. Dis.* 25: 7, 1941.
4. Moore, I. E., Eagle, H., Mohr, Ch. F.: Biologic False Positive Serologic Tests for Syphilis; Suggested Method of Approach to Their Clinical Study, *J. A. M. A.* 115: 1602, 1940.
5. Lynch, F. W., Boynton, R. E., Kimball, A. C.: False Positive Serologic Reactions for Syphilis Due to Smallpox Vaccinations (Vaccinia), *J. A. M. A.* 117: 591, 1941.
6. Porro, T. J.: The Kahn Reaction With Serum of Different Animals, *J. Infect. Dis.* 53: 210, 1933.
7. Greene, R. A., Harding, H. B., Hudspeth, W. T., Pistor, W. J.: Reaction of Sera of Different Animals to Kahn, Kline, Idé, Eagle, and Laughlin Tests, *J. Lab. & Clin. Med.* 23: 763, 1938.
8. Kahn, R. L.: Verification Test in Serology of Syphilis, *J. Lab. and Clin. Med.* 28: 1175, 1943.
9. Kahn, R. L., Marcus, S., McDermott, E. B., and Adler, I.: Serologic (Non-Syphilitic) Reaction Approaching Universal Sensitivity, *J. Invest. Dermat.* 5: 459, 1942.
10. Barnett, C. W., Jones, R. B., and Kulehar, G. W.: Measurement of Reagin in Non-syphilitic Sera, *Proc. Soc. Exper. Biol. & Med.* 33: 214, 1935.
11. Sherwood, N. P., Bond, G. C., Canuteson, R. I.: On Possible Presence of Reagin-like Factor in Normal Human Serum, *Am. J. Syph., Gonorr. and Ven. Dis.* 25: 179, 1941.
12. Lund, H.: Titration of Traces of Reagin: Technique of Flocculation Using Maximal Serum Proportions With Secondary Recovery of Antigen, *Am. J. Syph., Gonorr. and Ven. Dis.* 26: 1, 1942.
13. Kolmer, J. A.: The Problem of Falsely Doubtful and Positive Reactions in the Serology of Syphilis, *Am. J. Pub. Health* 34: 510, 1944.

MEDICAL ILLUSTRATION

AN INEXPENSIVE METHOD FOR PUBLISHING PHOTOGRAPHS OF FLUORESCENCE PHENOMENA IN COLOR

FRANK H. J. FIGGE, PH.D., AND THOMAS E. HOFFMASTER, JR.
BALTIMORE, MD.

IN A PREVIOUS paper a method for recording fluorescence phenomena on Kodachrome film was described.² Although this method reproduces the various fluorescent colors, the cost of publishing many of these pictures by the three or four-color process is prohibitive. A method was, therefore, devised for photographing fluorescence on black and white film in such a manner that reproductions of fluorescence were obtained which were suitable for printing in color by the Ben Day process. The resulting illustrations are useful to depict one or possibly two fluorescent colors. If only one color is pertinent, it may be emphasized and accurately recorded by this relatively inexpensive method.

One of the requirements for superimposing a color on a black and white illustration by the Ben Day process is that the area to be colored must be almost white. This effect is produced by great overexposure of the negative to the fluorescent light of the color which it is desired to record or emphasize. For example, if red fluorescence is to be recorded, the negative is overexposed to the red fluorescence by the use of a red filter so that red fluorescent tissues are recorded as nearly opaque areas on the negative. On the positive print these show up as almost white fields upon which the corresponding color can be superimposed by the Ben Day method. The precise color of the fluorescence can be described to the engraver or printer in terms of conventional color charts or notations,³ or the color can be matched by an artist. Printers' inks can be selected to match this color and thus accurately represent the fluorescence.

The illustration of the red fluorescence of the harderian gland of the rat (Plate I) was chosen to permit comparison of this new inexpensive method with the Kodachrome four-color method described previously in the JOURNAL.² A picture of the fluorescence of the rat and the harderian gland (Fig. 2, Plate I) in black and white is also shown. This is even less expensive than the process by which the Ben Day color process is applied but not nearly as satisfactory. The added color for purposes of depicting or emphasizing the fluorescence in a certain color costs but little more (Fig. 4, Plate I). The general principles and precise steps necessary to produce these illustrations are outlined.

The ultraviolet light sources and general principles of the use of filters for photographing fluorescence have been described.^{1,2} For Fig. 1 the rat with the harderian gland exposed was arranged on black paper. A 100-watt near-ultraviolet lamp and two 100-watt tungsten bulbs were placed on each side. A 500-watt tungsten lamp on a movable reflector was used to illuminate from

From the Departments of Anatomy and Art, School of Medicine, University of Maryland.
This work was aided by grants from the Anna Fuller Fund for Cancer Research, the International Cancer Research Foundation, and the Bressler Alumni Research Fund.
Received for publication, July 6, 1944.

different angles during the exposure to eliminate shadows. All the lights were turned on and the Super XX film was exposed for 40 seconds at $f:45$ without a filter.

In Fig. 2 the rat was photographed in a similar manner, except that no visible light was used and a Corning filter No. 3060 Noviol was interposed between the film and the object. A Wratten 2A filter would have been equally satisfactory. The rat was thus illuminated by only two near-ultraviolet lamps, one on each side. The Super XX film was exposed for 4 minutes at $f:16$. This gave, in reality, a photograph of the fluorescence on black and white film which has been reproduced as such. It may be seen that this type of picture is not suitable for the illustration of the red fluorescence of the harderian gland because the gland appears too dark to superimpose the red on it.

To obtain the photograph of the rat in Fig. 3 a red filter (Wratten F29) was arranged in a Universal swivel clamp so that it could be rotated into position in front of the lens after the film had been partially exposed with visible light. The Super XX film was then exposed by using the tungsten lights as well as the two near-ultraviolet lights for 12 seconds at $f:45$. This was only one-third of the exposure time which had been used for Fig. 1. The visible lights were then turned off and the red filter moved over in front of the camera lens. The exposure was then continued for 45 minutes at $f:5.6$. The red filter permitted the light from the red fluorescent gland to pass through and thus overexposed this area on the negative. It also transmitted the less intense red from other areas and completed the proper exposure of these areas. During the entire exposure the film was shielded from near-ultraviolet light by a Corning filter No. 3060 Noviol placed behind the lens. The red Wratten F29 filter also prevented near-ultraviolet light from reaching the film during the last 45 minutes of the exposure. Duplicate prints of photographs were made to submit to the engraver. One of these was colored to indicate the areas where Ben Day color was to be applied. The other black and white copy was necessary to make the engraving upon which the Ben Day color was superimposed (Fig. 4).

Fluorescence of other colors may be recorded and illustrated in a similar manner by choosing appropriate filters and film. In general, the film should be relatively sensitive to the color of the fluorescence to be recorded. Panatomic X or Super XX are satisfactory for red. The color sensitivity spectrograms have been found helpful in selecting film for recording various colors.

PLATE I

Four photographs of a normal rat with the left harderian gland exposed by the removal of the eyeball. Eastman 5 by 7 inch view camera, $6\frac{1}{2}$ inch Goerz Dagor lens.

Fig. 1.—Illumination: four 100-watt stationary incandescent tungsten filament bulbs, one moving 500-watt incandescent tungsten filament at approximately 3 feet, and two G.E. BH 4 100-watt quartz mercury arc near-ultraviolet lamps. Super XX film; no filter. Exposure time, 40 seconds at $f:45$.

Fig. 2.—Same as Fig. 1, except visible lights turned off. Illumination: two G.E. BH 4 100-watt mercury arc near-ultraviolet lamps. Secondary or lens filter: Corning No. 3060 Noviol. Exposure time, 4 minutes at $f:16$.

Fig. 3.—First exposure illumination: visible light same as Fig. 1 for 12 seconds at $f:45$. Secondary filter: Corning No. 3060 Noviol. Second exposure illumination: two G.E. BH 4 near-ultraviolet lamps only. Additional secondary lens filter: Wratten F29. Exposure time, 45 minutes at $f:5.6$.

Fig. 4.—Duplicate of photograph in Fig. 3 with red fluorescence of harderian gland indicated by Ben Day process. Compare with Fig. 2A, Plate I, Figgé and Clarke, this Journal, 27: 1609, 1942.

Fig. 1.



Fig. 2.



The Wratten tricolor filter sets are ideal for transmitting fluorescent light of any given color to overexpose the film. The following will be found satisfactory for any definite blue, green, or red fluorescence:

Wratten No. 49 filter C4 for blue

Wratten No. 61 filter N for green

Wratten No. 29 filter F for red

Only in rare instances will it be necessary to use filters that transmit wider bands of light. A hand spectroscope is excellent for determining the fluorescent spectrum so that a filter which specifically transmits these wave lengths may be selected. The only advantage to be gained by such precision would be possibly to shorten the exposure time in some instances. The intensity of the fluorescent light is usually so low that the exposure time cannot be determined by means of an ordinary exposure meter. Even with an extremely sensitive meter this is somewhat impractical, because the exposure time is so long, and excessive overexposure to fluorescent light from most objects is almost impossible.

If it is necessary to depict two fluorescent colors on one object, the film may be overexposed to each color separately and the areas for each color indicated. The Ben Day printer can place the colors to correspond to those observed on the object. Another alternative would be to make two photographs to be printed together, each depicting the fluorescence of one primary color.

SUMMARY

A method for photographing fluorescence on black and white film is described. The resulting photograph may be published in color by the relatively inexpensive Ben Day process.

REFERENCES

1. Figge, F. H. J.: Near-Ultraviolet Rays and Fluorescence Phenomena as Aids to Discovery and Diagnosis in Medicine, *Bull. School Med. Univ. Maryland* 26: 165, 1942.
2. Figge, F. H. J., and Clarke, C. D.: The Basic Technique of Kodachrome Photography of Fluorescence Phenomena, *J. LAB. & CLIN. MED.* 27: 1606, 1942.
3. Munsell, A. H.: A Color Notation, Baltimore, 1941, The Hoffman Brothers Co., p. 74.

BOOK REVIEWS AND NOTICES

Medical Diagnosis. Applied Physical Diagnosis. By *Roscoe L. Pullen, A.B., M.D.*, Instructor in Medicine, Tulane University of Louisiana School of Medicine; Assistant Clinical Director, Charity Hospital of Louisiana at New Orleans; Formerly Fellow in Clinical Endocrinology, Duke University School of Medicine and Duke Hospital, Durham, N. C. With a foreword by *John H. Musser, B.S., M.D., F.A.C.P.*, Professor of Medicine, Tulane University of Louisiana School of Medicine; Senior Visiting Physician, Charity Hospital of Louisiana at New Orleans. W. B. Saunders Co., Philadelphia and London, 1944. Cloth with 1,106 pages. 584 illustrations, and 12 color plates.

This new textbook of physical diagnosis differs in at least two ways from other similar books now available. First, it was written by twenty-six clinicians in addition to the editor; there are many evidences, therefore, of the specialized information one would expect when authorship is the work of a group of physicians rather than of one. Second, it devotes chapters to techniques of examinations which are usually treated very briefly in textbooks of physical examination. There are, for instance, detailed discussions of proper methods for examining the skin, the eyes, the ears, nose, and throat, the oral cavity, the breasts, the urogenital tract, and the anorectal region. Methods for making an endocrine survey are described. Chapters are devoted to psychiatric diagnosis, to special problems of pediatric diagnosis, to occupational injuries, and to military problems. Endoscopic techniques, like proctoscopy, are described in considerable detail. Description of these methods gives the book special value to physicians as a reference source. Hospital house staffs should find them most useful.

In most of the sections, material is presented in a very readable style. Illustrations are clear, well chosen, and really instructive. The chapter on the examination of the extremities is particularly well done. One wonders, however, at the wisdom of providing medical students with a textbook on physical diagnosis which devotes only sixty-four pages to examination of the chest, but forty-five to examination of the breasts and ninety-eight to gynecologic and obstetric diagnosis. There are certain other inconsistencies. Thirteen positions are given in which the breasts may be studied, but no mention is made of the fact that spleens may often be felt more readily when a patient lies on his right side with the left thigh flexed on the abdomen. The inclusion of histologic findings under conditions where the changes do not contribute to an understanding of the attendant physical signs is disconcerting. The same objection applies to the listing of certain hematologic data in a discussion of enlarged lymph nodes.

These criticisms, however, are not meant to imply that the book is not an unusually serviceable one. It will undoubtedly be adopted as the standard text in many medical schools. For the general practitioner, the internist, and the surgeon, the special chapters already mentioned will be particularly valuable guides.

The Journal of Laboratory and Clinical Medicine

VOL. 29

DECEMBER, 1944

No. 12

CLINICAL AND EXPERIMENTAL

STUDIES IN HUMAN INHERITANCE

XXV. IS THE HOMOZYGOUS FORM OF MULTIPLE TELANGIECTASIA LETHAL?

LAURENCE H. SNYDER, Sc.D.,* AND CHARLES A. DOAN, M.D.†
COLUMBUS, OHIO

MULTIPLE telangiectasia has long been known as a genetic trait, dependent upon a dominant hereditary factor. The first family history was presented in 1876 by Legg. Rendu, in 1896, was the first to associate the presence of epistaxis with telangiectatic capillary tufts as a clinical manifestation of a distinct morbid entity, and Osler further elaborated on this syndrome five years later.

From time to time summaries of the reported families have appeared, all testifying to the dominant nature of the gene concerned. Hanes, in 1909, reviewed fifteen families, including two of his own. Steiner, in 1917, recorded twenty-five published cases and added three more. In 1923 Fitz-Hugh was able to summarize 212 cases in thirty families, and by 1926 East listed thirty-five families. By 1930 there were on record 550 members of ninety families, and by 1932, about 600 members of 100 families (Goldstein, 1931, 1932). At the present time there are certainly more than a thousand cases on record, belonging to about 150 families.

Figi and Watkins, in reviewing the Mayo Clinic material recently (1943), state that "while the condition at times gives rise to fatal epistaxis, in general it does not inhibit development or shorten the span of life." The sexes are equally affected, and in all but a very few instances the trait is obviously present in one of the parents of an affected individual. There have been occasional

*Chairman, Department of Zoology and Entomology, and Professor of Medical Genetics, Department of Medicine, the Ohio State University.

†Professor and Chairman, Department of Medicine, and Director of Medical Research, the Ohio State University.

Received for publication, Aug. 19, 1944.

instances where neither parent has shown the telangiectasia (Cockayne, 1933), but these appear to be the result of internal manifestations only or mutation (Roberts, 1940).

The criteria for diagnosis are generally agreed to be definite, hereditary, visible telangiectasia, usually of the nose, tongue, lips, face, or fingers, and a tendency to bleed from the lesions spontaneously or on minimal irritation (Larrabee and Littman, 1932). However, the lesions may be numerous or very few, or occasionally none may be found at all (Roberts, 1940). Roberts reports a case in which the attacks of epistaxis and the family history were typical, but a careful examination revealed only a single visible hemangioma which was upon the palate. He also records among thirteen new cases one mutation to multiple telangiectasia, the other twelve cases presenting family histories typical of the dominant heredity.

A gene which is completely dominant produces the same effect when heterozygous (that is, present only once; inherited from an affected parent, the other gene being the normal allele) as when homozygous (present in duplicate; inherited from two affected parents). The gene for telangiectasia could be homozygous in any individual only if both parents had the dyscrasia and each transmitted the gene to that individual. Nowhere, to our knowledge, has there been recorded an instance of the marriage of two persons exhibiting multiple telangiectasia. In all instances, at least one of the parents has been normal, so that we know only the heterozygous form of the condition and have had no opportunity up to now to observe the homozygous form.

It has hitherto been assumed that the gene for multiple telangiectasia is completely dominant. But we have no right to say a priori that the homozygous state would be similar to the known heterozygous condition. Genetics is replete with examples of genes which, while resulting in viable deviations from normal in the heterozygous condition, are lethal in homozygous form.

In man such genes are known or strongly suspected in minor brachydactyly (Mohr and Wriedt, 1919), sebaceous cysts (Munro, 1938), and certain types of mental defects (Penrose, 1938). The name semidominant lethal has been given to genes of this nature by one of us (Snyder, 1940) to distinguish them from typical dominant genes on the one hand and from recessive lethal genes which produce no visible effect in the heterozygous state on the other.

There has recently come before us a case (Fig. 1) which would indicate that the gene for multiple telangiectasia is a semidominant lethal gene, the homozygous state of which is incompatible with life. This case is, to the best of our knowledge, the first recorded instance of an offspring with two proved telangiectatic parents.

CASE REPORT

E. A. P., an 8-pound white female infant, was born at term by low forceps delivery Oct. 23, 1942, in White Cross Hospital, Columbus, Ohio, the first child of young parents. At the time of birth the baby appeared normal in every respect, except for an area approximately 5 cm. in diameter over the left breast and clavicle in which the superficial vascular bed was strikingly dilated. In addition to the prominent larger branches of the vascular tree, the arterioles, capillaries, and venules were diffusely dilated, giving an overall hyperemia which could be readily obliterated by light pressure over the area. There was no ulceration and no other skin or mucous membrane blemish. The attending obstetrician explained this to

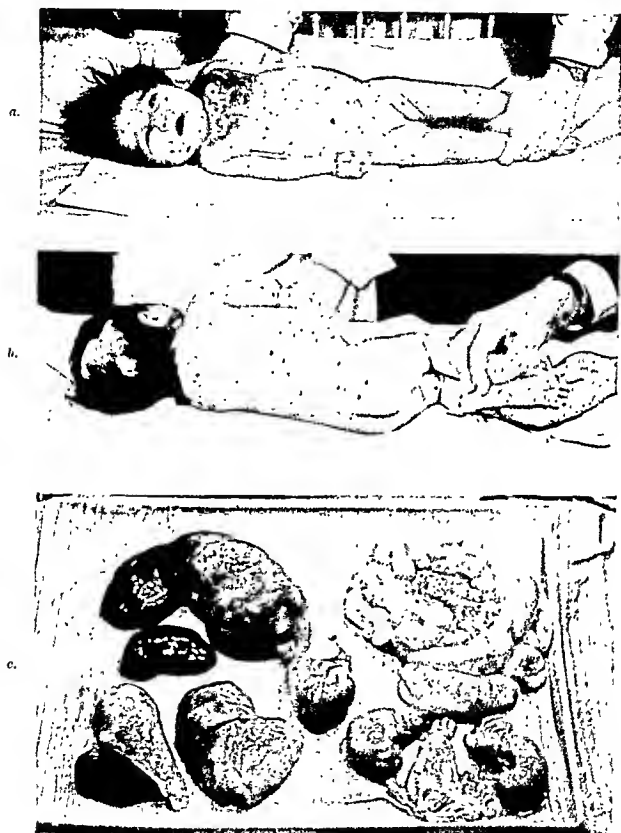


Fig. 1—*a*, Generalized multiple telangiectasia in infant 10 weeks, 4 days of age. Note large ulcerated area involving the neck and left chest.

b, The hemangiomas stand out prominently, surrounded by a lighter halo against a background of generalized diffuse cyanosis during crying episodes.

c, All internal organs, including the brain, showed multiple hemangiomas and hemorrhage.

the parents as a "port-wine birthmark," which could be treated at the proper time and which need cause no immediate concern. The mother and baby were discharged from the hospital to their home on the second day.

On the fifth day after birth the mother noticed for the first time the appearance of three small slightly raised "red spots" on the infant's right cheek near the corner of the mouth. By the ninth day five small, pinhead sized, raised, red areas were present on the face, and several similar lesions were noted on each hand, the infant continued to be mentally alert and was physically well developed and well nourished. On the twenty-second day the infant had developed both vertical and rotary eye coordination and would fix her vision on a light or any bright object.

By November 18, when the baby was 26 days old, these red "purpuric" areas had spread sufficiently so that the attending physician referred the patient to one of us (C. A. D.). When seen at this time the infant was well nourished, alert, and in no apparent pain or distress. The formula was being taken satisfactorily and a satisfactory weight gain had been recorded since birth. The skin and mucous membranes did not suggest any anemia; however, scattered sparsely over these surfaces were flat or slightly raised, sharply circumscribed, bright cherry red subcutaneous areas, ranging in size from 1 to 5 mm., and in the region of the left clavicle was a similar but much larger diffusely hyperemic, purple-red lesion under a very thin superficial layer of epithelium. Both large and small lesions could be readily and completely obliterated by light pressure. There were no true petechiae or ecchymoses demonstrated anywhere. A detailed analysis, particularly of the larger lesions, left no doubt as to the nature and extent of the engorgement and dilatation of localized capillary tufts responsible for the findings. There was no generalized lymphadenopathy. The heart and lungs were normal to physical examination. Neither spleen nor liver could be palpated, and there were no intra-abdominal masses. Neurologic findings were physiologic.

Laboratory data at this time showed normal urine and stool; total white blood cells, 10,500; total red blood cells, 4,760,000; hemoglobin, 12.8 Gm.; reticulocytes, 0.6 per cent; blood platelets, 932,960 per cubic millimeter, supravital differential of the white blood cells: polymorphonuclear neutrophils, mature, actively motile, 35 per cent; eosinophiles, 1 per cent; lymphocytes, small, mature, 59 per cent; monocytes, mature, 5 per cent. There were no significant qualitative abnormalities seen in any of the circulating elements. Bleeding and coagulation times were normal. A diagnosis was made of "hereditary hemorrhagic telangiectasia," of Rendu-Osler type, without as yet any hemorrhagic manifestations. The potential danger of hemorrhage from any skin or mucous membrane lesion was explained to the mother.

At 28 days of age the first signs of hemorrhage appeared in urine and stool. The infant became more fretful and wakeful, and her increased activity and fingernail scratching of the large superficial subclavicular birth lesion resulted in a breakdown of the skin with a slow oozing of blood difficult to control. The telangiectatic foci became suddenly much more numerous on face, hands, arms, legs, palms, and soles of feet. A low-grade fever developed and she refused her formula. During crying spells the entire skin of the body and the mucous membranes would develop a reddish purple cyanosis, with the superimposed deeper telangiectatic foci prominently and numerous highlighted (Fig. 1, b).

A progressive downhill clinical course continued at home until Jan. 1, 1943, when the baby was admitted to the Hematology Service at University Hospital because of increasing weakness, pallor, fever, insomnia, anorexia, vomiting, constipation alternating with bloody diarrhea, hematuria, and apparent loss of vision. Admission weight was 9 pounds; temperature, 101° F. (rectally); pulse, 140; respiration, 26. The skin and mucous membranes at this time were very pale and were literally peppered everywhere, including the scalp, with enlarged capillary hemangiomas ranging in size from 5 mm. to 1 cm. (Fig. 1, a). The skin over the large birth lesion had completely broken down and was covered with a blood clot. Vision was completely lost due to bilateral retinal hemorrhages, and periodically the infant would suddenly cry out as though from sudden pain. No new findings were noted on physical examination except extreme pallor alternating with a generalized dusky cyanosis when crying (Fig. 1, b) and a marked, diffuse, precordial, systolic murmur presumably hemic in origin. Hematology—total white blood cells, 27,800; total red blood cells, 1,770,000; hemoglobin, 6.1 Gm.; reticulocytes, 1.0 per cent; blood platelets, 297,360 per cubic

millimeter; supravital differential of the white blood cells: polymorphonuclear neutrophils, motile, mature, segmented, 37 per cent; nonsegmented, 31 per cent; metamyelocytes, 1 per cent; myelocytes "C," 3 per cent; basophiles, 1 per cent; eosinophiles, 0; lymphocytes, small, mature, 23 per cent; monocytes, 4 per cent. Urine, negative for albumin and sugar. Microscopic 2-3 red blood cells per high-power field. Feces, occult blood *positive*.

Under observation new hemangiomas were seen to develop each day, and existing foci tended to enlarge and in some instances coalesce with adjacent tufts. Kodachrome records were obtained which showed the lesions before and during crying with the development of the generalized cyanosis with diffuse widespread capillary bed dilatation already mentioned. The formula was adjusted and taken and retained, orange juice and cod-liver oil were added, but otherwise little change was noted.

When the child was discharged from the hospital Jan. 5, 1943, the hematologic data were as follows: total white blood cells, 21,300; total red blood cells, 1,440,000; hemoglobin, 2.3 Gm.; reticulocytes, 0.8 per cent; blood platelets, 406,080 per cubic millimeter; supravital differential of the white blood cells: polymorphonuclear neutrophils, mature, actively motile, 71 per cent; metamyelocytes, 2 per cent; myelocytes "C," 2 per cent; lymphocytes, small, 7 per cent; monocytes, 18 per cent.

The patient's condition became steadily worse and death occurred at home 6:00 A.M., Jan. 10, 1943, at the age 2 months, 18 days. A post-mortem examination was made at the University Hospital 10:00 A.M. the same date. The body weighed 3,450 Gm. The anatomic findings and the final histopathologic diagnosis included general malnutrition, multiple hemangio-endotheliomas, telangiectases of the skin, mucous membranes, lungs, spleen, liver, intestines, kidneys, and brain; ulceration of large telangiectatic area on the upper left chest; pulmonary congestion, edema, and atelectasis; congestion of adrenals, kidneys, and brain; hyperplastic red bone marrow. In summary, this infant exhibited a fundamental deficiency involving solely the vascular tree, more particularly the capillary bed and small venules, with the development in utero of one large localized area of pathologic dilatation over the left chest. Shortly after birth this vascular defect further manifested itself through the rapid development of multiple telangiectatic hemangio-endotheliomas involving the entire superficial skin and mucous membranes and the majority of the internal organs. Both superficial and internal hemorrhages occurred, speedily interfering with normal gastrointestinal and urinary functions, with a fatal outcome due principally to the profound anemia which resulted.

DISCUSSION

The appearance of the infant when first seen by us, covered as she was with multiple, raised, red areas which blanched or completely disappeared under pressure, immediately suggested fulminant generalized telangiectasia. Since the usual onset is during childhood or adolescence, and since the usual occurrence of lesions is far less extensive and severe than in this instance, it seemed possible that this case might represent the homozygous manifestation of the gene previously known only in the heterozygous form.

Accordingly the family was carefully investigated for the occurrence of telangiectasia. Fortunately, in addition to the parents all four grandparents were living, readily available, and fully cooperative. Examination revealed that both parents, the maternal grandfather, and the paternal grandmother exhibited classical superficial telangiectatic foci. Neither parent had any past personal history of epistaxis or any unusual bleeding tendency. Both had always been in good health, and the father, at the time of the birth of his daughter, was serving in the U. S. Army. On physical examination eight small (from 5 to 25 mm. in diameter) telangiectatic foci were found scattered over the surface of the skin of the mother and three on the father. No mucous membrane lesions were found, and the readily obliterated telangiectatic capillary tufts in the skin showed no tendency to enlarge or hemorrhage.

The maternal grandfather of the patient, 42 years of age, reported frequent spontaneous epistaxes following excessive exercise or overheating up to 16 years of age. Since then they have occurred much less frequently. On inspection fifteen telangiectatic foci were readily found from (5 to 15 mm. in diameter) on face, shoulders, abdominal wall, and buttocks; none could be seen in the mucous membranes of the nose or nasopharynx. The paternal grandmother, 60 years of age, while having no past personal history of hemorrhage, showed twelve typical telangiectatic tufts (from 5 mm. to 50 mm. in diameter) scattered subcutaneously over the torso, some raised, others flat, but all readily obliterated on pressure; no mucous membrane lesions were seen.

One paternal uncle of the patient is said to be a "bleeder," having experienced a near-fatal hemorrhage at birth. This individual is married and has a normal child but has not been available for direct questioning or examination. Three other paternal granduncles have rather severe diabetes mellitus.

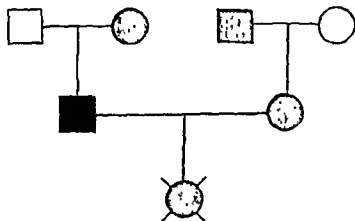


Fig. 2.—Family history of the patient. Solid squares and circles represent classical telangiectasia, solid circle with projecting spokes represents the patient, who apparently had the homozygous lethal form of the dyscrasia. The patient died at 11 weeks of age.

The family history is presented in Fig. 2. Inspection of the pedigree will at once reveal the fact that multiple telangiectasia appeared in both sides of the family, being transmitted in typical heterozygous form until it reached the infant under discussion. Here the opportunity was presented for the gene to be inherited from both parents and to occur in the homozygous state. The added facts that the trait was first manifested during fetal life, followed by the rapid development of multiple telangiectasia during the first weeks of life, and that the progress and extent of the vascular defect were far more severe than the usual manifestations, terminating in early death, support the correctness of the assumption that this infant represents the expression of the homozygous state of the gene.

The question of the occurrence of the same condition in future children logically arises. Letting T' represent the semidominant lethal gene for telangiectasia and t represent its recessive normal allele, three genotypes are possible. They are $T'T'$, resulting in the lethal form of the trait; Tt , resulting in classical telangiectasia; and tt , resulting in the absence of the dyscrasia.

The parents of the infant were obviously each of the genotype Tt . Their offspring would be expected to occur in the ratio one $T'T'$: two Tt : one tt . The chances of another child being affected with the lethal form of the dyscrasia

are thus one in four. The chances of a child in this family having classical telangiectasia would be two in four, and of a child being completely unaffected, one in four.

It may be added that there are on record a number of instances of generalized telangiectasia in adults. These differ markedly from the present case in several respects. The onset is very much later, the telangiectasia are far less extensive, no family history is presented, and the condition is entirely compatible with life. Examples of adult-acquired generalized telangiectasia are those of Osler (1907) and Ormsby and Mitchell (1922).

Osler's patient was a man of 39, with generalized telangiectasia of trunk and extremities of about ten years' duration. The family history was negative. The patient of Ormsby and Mitchell was a 33-year-old woman with a generalized telangiectasia of twenty years' duration, found on face, forehead, neck, chest, back, arms, fingers, and palms.

SUMMARY

A case is presented of rapidly developing, severe, generalized, multiple telangiectasia in a newborn female infant. The condition, beginning in utero, was incompatible with life, death occurring at 11 weeks of age. Classical telangiectatic hemangiomas occurred in the paternal grandmother, the maternal grandfather, and both parents. The family history suggests the probability that this case represents the first recorded instance of the homozygous form of the dyscrasia and that the gene for multiple telangiectasia is lethal when homozygous.

REFERENCES

- Cockayne, E. A.: *Inherited Abnormalities of the Skin and Its Appendages*, London, 1933, Oxford University Press.
- East, C. F. T.: *Familial Telangiectasia*, *Lancet* 1: 332, 1926.
- Figi, F. A., and Watkins, C. H.: *Hereditary Hemorrhagic Telangiectasia*, *Ann. Otol. Rhin. & Laryng.* 52: 330, 1943; Abstracted in *Proc. Staff Meet., Mayo Clin.* 18: 418, 1943.
- Fitz-Hugh, T., Jr.: *Importance of Atavism in Diagnosis of Hereditary Telangiectasia*, *Am. J. Med. Sc.* 166: 884, 1923.
- Goldstein, H. I.: *Hereditary Multiple Telangiectasia*, *Goldstein's Heredofamilial Angiomatosis With Familial Telangiectasia*, *Osler-Weber's Disease*, *Arch. Dermat. & Syph.* 26: 282, 1932.
- Hanes, F. M.: *Bull. Johns Hopkins Hosp.* 20: 33, 1909.
- Larrabee, R. C., and Littman, D.: *Hereditary Hemorrhagic Telangiectasia, With Report of Five Cases in Two Families*, *New England J. Med.* 207: 1177, 1932.
- Legg: *Lancet* 2: 856, 1876.
- Mohr, O. L., and Wriedt, C.: *A New Type of Hereditary Brachyphalangy in Man*, *Carnegie Inst. of Wash., Pub.* 295, 1919.
- Munro, T. A.: *Hereditary Sebaceous Cysts*, *J. Genetics* 35: 31, 1937.
- Ormsby and Mitchell: *Chicago Dermatological Society Transactions, Abstract*, *Arch. Dermat. & Syph.* 5: 781, 1922.
- Osler, W.: *On a Family Form of Recurring Epistaxis, Associated With Multiple Telangiectases of the Skin and Mucous Membranes*, *Bull. Johns Hopkins Hosp.* 12: 333, 1901; *On Telangiectasis Circumscripta Universalis*, 18: 401, 1907.
- Penrose, L. S.: *Some Genetical Problems in Mental Deficiency*, *J. Ment. Sc.* 84: 693, 1938.
- Rendu: *Gaz. des hopitaux* 49: 1322, 1896.
- Roberts, J. A. F.: *An Introduction to Medical Genetics*, 1940, London, Oxford University Press.
- Snyder, L. H.: *The Principles of Heredity*, ed. 2, Boston, 1940, D. C. Heath & Co.
- Steiner, W. R.: *Hereditary Hemorrhagic Telangiectasia*, *Arch. Int. Med.* 19: 194, 1917.

SYMPATHOLYTIC TREATMENT OF EXPERIMENTAL HYPERTENSION

JOHN JACOBS, M.D., AND FREDRICK F. YONKMAN, PH.D., M.D.
DETROIT, MICH.

IN APRIL, 1940,¹ the following statement¹ was made with reference to desirable therapy for essential hypertension: "Theoretically, a chemical substance capable of neutralizing or inhibiting the action of any vasoconstricting agent, whether it be epinephrine, pituitrin, renin, or sympathin, would be the agent of choice. . . . By this means we may probably treat essential hypertension." This statement was predicated upon experimental results obtained with yohimbine hydrochloride and ethyl yohimbine hydrochloride which demonstrated the antisympathicomimetic potentialities of these agents. They

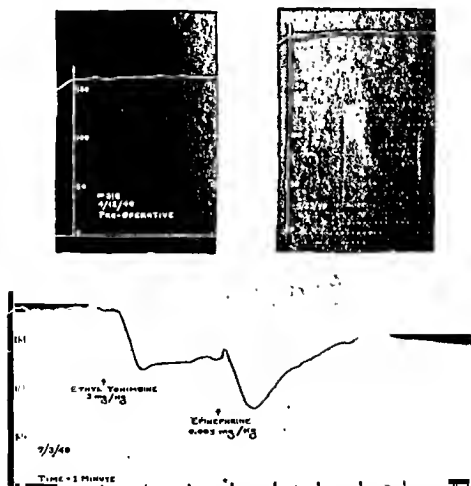


FIG. 1.—Arterial tension of a dog recorded by arterial puncture and expressed in millimeters of mercury. Note that tension is elevated postoperatively but is decreased by ethyl yohimbine, 3 mg. per kilogram, and that the normal hypertensive effect of epinephrine, 0.005 mg. per kilogram, is reversed by the yohimbine radicle. (Courtesy Chase, Yonkman, and Lehman.)

are antiadrenergic at vasoconstrictor foci² and are both adrenolytic and sympatholytic for salivation and mydriasis.³ The vascular "epinephrine-reversal" effect is maintained under these yohimbine salts even in the hypertensive

From the Department of Pharmacology and Therapeutics, Wayne University.

Received for publication, April 7, 1941.

state,⁴ and it was this fact (Fig. 1) which prompted the present study. It was assumed that if yohimbine and its congeners paralyzed sympathetic control of the vascular bed after intravenous administration, the same anti-sympathetic action might be obtained in hypertensive animals after prolonged oral feeding of the drugs. The result then should become manifest by a gradual reduction in blood pressure.

METHOD

Dogs of both sexes, averaging about 20 Kg. in weight, were anesthetized with pentobarbital given intravenously, and their kidneys were encased by the Page⁵ technique, using oiled silk for envelopes instead of cellophane, since the latter was too prone to become fragmented before an appreciable degree of constricting fibrocollagenous deposition had been effected perirennally. These envelopes were prepared in advance with purse string sutures so as to anchor the jacket loosely around the hilus. They were then autoclaved and kept sterile.

Systolic and diastolic blood pressures were recorded at intervals of from five to seven days from the brachial artery with a pediatric cuff, stethoscope,

TABLE I
EXPERIMENTAL HYPERTENSION IN DOGS
(MEAN BLOOD PRESSURES IN MILLIMETERS OF MERCURY)

ANIMALS	NORMAL	MAXIMUM*	MINIMUM FOLLOWING MAXIMUM*	STABILIZED*	FINAL	VALUE OF TREATMENT IN PERCENTAGE OF PRESSURE REDUCTION	REMARKS
<i>Controls</i>							
Group A	99	---	---	---	100		Dummy operation; kidneys stripped but not wrapped
Group B	98	160	149	152	152		Operated but unmedicated
Group C	103	142	137	137	137		Operated but unmedicated
<i>Medicated</i>							
Group D	101	165	159	160	121	24	Received 20 mg. per kilogram of yohimbine hydrochloride for 35 days
Group E	99	152	141	144	132	8	Received 20 mg. per kilogram of yohimbine hydrochloride for 33 days
Group F	102	148	145	147	142	3	Received 20 mg. per kilogram of yohimbine hydrochloride for 23 days
Group G	97	156	150	151	151	--	Received 20 mg. per kilogram of yohimbine hydrochloride for 17 days

*Prior to treatment.

and aneroid manometer.⁶ All control and experimental pressures were read by the same individual after the dogs were trained to mount a stairway and lie quietly unrestrained for from fifteen to thirty minutes upon a padded table.

Food, water, and exercise were permitted *ad libitum*. Medication consisted of yohimbine hydrochloride⁴ in a dose of 20 mg. per kilogram daily; it was administered orally in capsule form for varying periods of time after the hypertensive level had become stabilized for ten days or more.

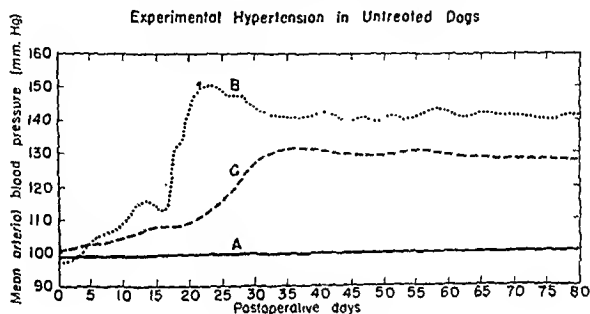


FIG. 2

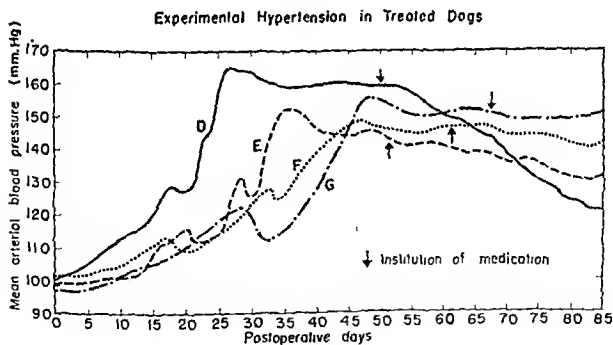


FIG. 3.

RESULTS

More than twenty dogs were employed in this project but some failed to survive long enough to be of much value from the point of view of evaluating treatment. This was especially true for those animals in which both kidneys had been encapsulated. Hypertension developed so rapidly and so severely in these that death not infrequently occurred in three or four weeks after surgery. Hence, unilateral envelopment was resorted to. Table I presents the

⁴Adequate supplies were made available by Metek & Co., Inc., Rahway, N. J.

data on seven dogs, three of which served as controls and four as experimental subjects. One of the control dogs (A) was operated in the same manner as the other dogs except that after the perirenal adnexa had been stripped, no oiled silk envelopes were placed around the kidneys. This animal's mean blood pressure remained stabilized at approximately 100 mm. Hg. Dogs B and C became hypertensive following renal encasement, their mean blood pressures stabilized at 152 and 137 mm. Hg, respectively. They received no treatment and served as controls for the medicated animals, Dogs D, E, F, and G, whose pressures stabilized at 160, 144, 147, and 151 mm. Hg, respectively. Reduction of blood pressure occurred in three of these medicated hypertensive dogs, but in only two (D and E) was the decrease of significance, 24 per cent reduction in Dog D and 8 per cent in Dog E. It is problematic whether sustained medication might have effected further pressure reduction in all dogs, especially in E and F. This could not be determined.* Data are charted in Figs. 2 and 3.

Initially, the total dose of 20 mg. per kilogram of yohimbine hydrochloride was given in one administration. This brought on severe symptoms of weakness, incoordination, partial paralysis, salivation, panting, general restlessness, and suggestions of apprehension from about thirty to forty minutes after ingestion. These reactions are typical of acute hypotension; they endured for about forty-five or sixty minutes, and their severity necessitated division of the medication into three daily doses of 7 mg. per kilogram each. Later, two doses of 10 mg. per kilogram were given, and, finally, the original 20 mg. dosage was well tolerated. Hence, it seems that a degree of tolerance is developed to certain actions of this alkaloid.

DISCUSSION

Hypertension developed by the Page technique can be reduced by an anti-adrenergic vasodilator such as yohimbine. This reduction is not uniformly produced and it varies in degree with this type of therapy. Final conclusions regarding the evaluation of such treatment in terms of the percentage drop in blood pressure per animal as well as the incidence of significant decreases in the group treated await further investigation.

The pharmacologic action of yohimbine in this situation has as yet not been determined. Whether some break in "Page's humoral sequence" is produced by release of renal ischemia, inhibition of renin, or dampening of angiotonin's effect is a moot point. It can be stated, however, that the latter does not seem probable, since in acute vascular studies⁷ yohimbine does not interfere with the normal vasoconstrictor action of angiotonin. Thus, it would seem plausible that vasoconstrictor tone may be lessened, either by a direct nitritelike action or, more probably, by an antiadrenergic or sympatholytic action in relation to neural vasoconstrictor control of blood vessels.^{3,7} Epinephrine-reversal experiments in this series of hypertensive dogs were not done because of the hazards associated with acute hypotension induced by this procedure. This experiment is contemplated in our next series and further light on the nature of yohimbine's action in this hypertensive state should be forthcoming.

*John Jacobs entered the U. S. Army Aug. 16, 1943.

SUMMARY

1. Yohimbine hydrochloride, 20 mg. per kilogram when orally administered, reduces the mean arterial tension of dogs rendered hypertensive by the Page technique of perirenal envelopment.

2. Reduction of arterial tension was effected in three of four dogs. One was of real significance, another of some, and the third was inconsequential; these appraisals were made after thirty-five, thirty-three, and twenty-three days of medication, respectively. Sustained medication for longer periods would be desirable.

3. The locus of yohimbine's pharmacologic action in this type of hypertension is probably in the neuromuscular receptors associated with sympathetic vasoconstrictors and is probably antiadrenergic or sympatholytic in nature.

REFERENCES

1. Yonkman, F. F.: *The Challenge*, J. Wayne Univ. Col. of Med. 3: 4, 1940.
2. Hamet, R.: On a New Instance of the Inversion of the Effect of Adrenaline, *Compt. rend. Acad. d. sc.* 180: 2074, 1925.
3. Yonkman, F. F., and Young, A. G.: The Antisymphathicomimetic Effect of Ethyl Yohimbine on Salivation and Mydriasis, *J. Pharmacol. & Exper. Therap.* 63: 40, 1938.
4. Chase, H. F., Yonkman, F. F., and Lehman, A. J.: The Effect of Ethyl Yohimbine in Experimental Hypertension, *J. Pharmacol. & Exper. Therap.* 72: 6, 1941.
5. Page, I. H.: Production of Persistent Arterial Hypertension by Cellophane Perinephritis, *J. A. M. A.* 113: 2046, 1939.
6. Allen, F. M.: Auscultatory Blood Pressure Methods for Dogs, *J. Lab. & Clin. Med.* 27: 371, 1941.
7. Yonkman, F. F., Jeremias, R., and Stilwell, D.: Angiotonin Myotropism, *Proc. Soc. Exper. Biol. & Med.* 54: 204, 1943.

TOXICITY OF YOHIMBINE HYDROCHLORIDE

FREDRICK F. YONKMAN, PH.D., M.D.
DETROIT, MICH.

YOHIMBINE is said to be less toxic than cocaine,¹ but data regarding chronic toxicity during prolonged feeding are not available. Procurement of these data seemed desirable because of the potential value of this alkaloid in experimental treatment of hypertension, particularly of the neurogenic type. It is conceivable that vascular spasticity may be released by yohimbine since this drug is sympatholytic² as well as adrenolytic.³ Should the drug be too toxic, however, clinical trial would have to be discouraged despite the nicety of one's therapeutic rationale.

METHOD

Young white rats of both sexes were divided into four groups of four each with at least one male or female appearing in each group for breeding purposes during the first experiment. Sexes were separated in the four groups utilized for the second experiment. A basic ration consisting of Steenbock's diet was fed *ad libitum*, and yohimbine hydrochloride* was added to the drinking water bottles and was likewise permitted *ad libitum*. Control rats (Groups A and E) received tap water and experimental rats (Tables I and II) received yohimbine in tap water as follows: Group B, 1:40,000; Group C, 1:80,000; Group D, 1:54,000; Group F, 1:10,000; Group G, 1:5,000; and Group H, 1:1,000. Fresh solutions of yohimbine were made every two or three days because of the supposed instability of the solution.¹ All rats were weighed individually at six- or seven-day intervals, but the average weight of each group of animals was chosen for charting purposes in presenting the growth curves. Careful watch was made for any signs or symptoms which might indicate toxicity, and the average daily water consumption was determined on two occasions, six weeks apart.

One animal selected at random from Groups A, B, C, D, E, F, G and the lone surviving animal in group H were autopsied by Dr. Mark Maun, of the Department of Pathology.

RESULTS

Growth curves of the experimental rats in the first study, which lasted for thirteen weeks (Fig. 1), indicate that the growth of animals which were permitted access to yohimbine in concentrations of 1:80,000, 1:54,000 and 1:40,000 compared favorably with those shown by the controls. Reproduction was ap-

*Yohimbine hydrochloride was generously supplied to us by Merck & Co., Inc., Rahway, N. J., and will be referred to in this paper as yohimbine.

From the Department of Pharmacology and Therapeutics, Wayne University College of Medicine.

Acknowledgment is hereby made to Mr. Elmer Fisher, technician, for his cooperation in this project.

Received for publication, May 8, 1944.

TABLE I

YOHIMBINE FEEDING STUDIES IN WHITE RATS
(Feeding Begun 11/13/41; Autopsies Performed 2/16/42)

WEEKS	GROUPS (WEIGHT IN GM.)				REMARKS
	A	B	C	D	
	CONTROLS	1:40,000*	1:80,000*	1:54,000*	
1	127	107	118	108	
2	167	112	126	114	
3	172	143	153	140	
4	189	155	171	142	
5	208	168	197	153	
6	242	204	206	184	
7	253	208	210	196	
8	252	209	222	221	
9	266	221	229	190	2 of 4 rats in Group D had normal litters
10	265	203	209	205	1 of 4 rats in Group B had a normal litter
11	247	186	211	219	1 of 4 rats in Group A had a normal litter
12	254	217	237	223	
13	259	229	242	220	1 of 4 rats in Group D had a normal litter

*Concentration of yohimbine in drinking water.

TABLE II

YOHIMBINE FEEDING STUDIES IN WHITE RATS
(Feeding Begun 2/13/42; Autopsies Performed 5/25/42)

WEEKS	GROUPS (WEIGHT IN GM.)				REMARKS
	E	F	G	H	
	CONTROLS	1:10,000*	1:5,000*	1:1,000*	
1	61	55	69	60	
2	50 c.c.	33 c.c.	27 c.c.	6 c.c.	Daily water consumption per rat
3	63	49	58	43	
4	74	69	76	64	2 of 4 animals died; rats in groups F, G, and H all drink less water but eat more than control rats
5	103	91	93	68	
6	119	103	99	63	
7	142	105	107	80	Third of 4 rats died
8	48 c.c.	35 c.c.	30 c.c.	5 c.c.	Daily water consumption per rat
9	171	126	141	55	
10	182	134	142	60	
11	198	138	157	63	
12	230	147	171	66	
13	241	178	180	85	
14	247	179	183	87	
15	228	179	191	100	
16	214	188	181	98	

*Concentration of yohimbine in drinking water.

parently not interfered with, since in two groups, B and D, litters appeared as in control Group A. In Group D a total of three litters appeared. All rats seemed to be in good health and their habits were normal.

Growth curves of the experimental rats in the second study, which extended through fourteen weeks (Fig. 2), indicate that yohimbine in concentrations of 1:10,000 and 1:5,000 were quite well tolerated, but a concentration of 1:1,000 was definitely detrimental to growth. In the latter group (H, 1:1,000) two of four rats died during the third week and another during the fifth week of the experiment. Water consumption studies revealed that less water was consumed as the concentration of yohimbine was increased. At no time did the members of Group H appear at full vigor, and members of Groups F and G seemed to have lost some of their usual vigor during the last three or four weeks.

Gross and microscopic examination revealed no significant deviation from the normal. In only one animal (the lone survivor of Group II, 1:1,000 concentration of yohimbine) were there any aberrant findings which might be attributed to the medication. The pathologic report follows:

YOHIMBINE FEEDING STUDIES IN WHITE RATS

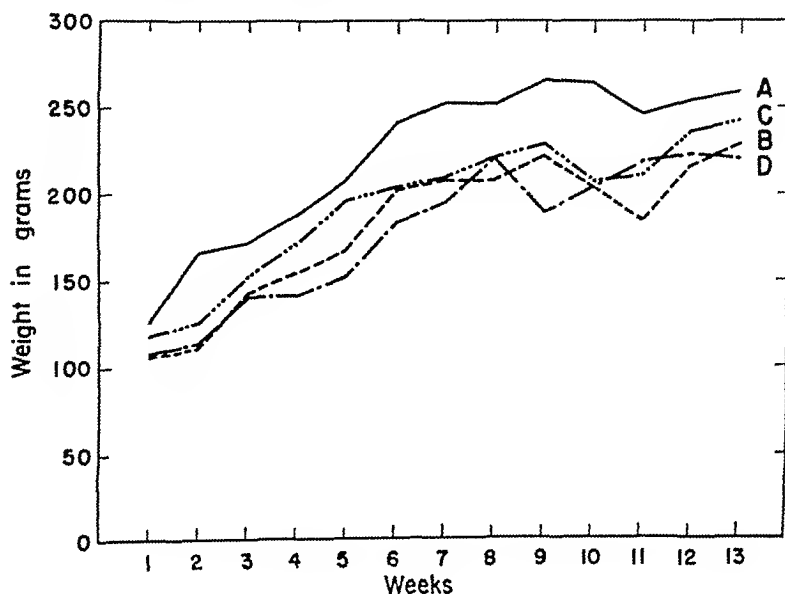


Fig. 1.

YOHIMBINE FEEDING STUDIES IN WHITE RATS

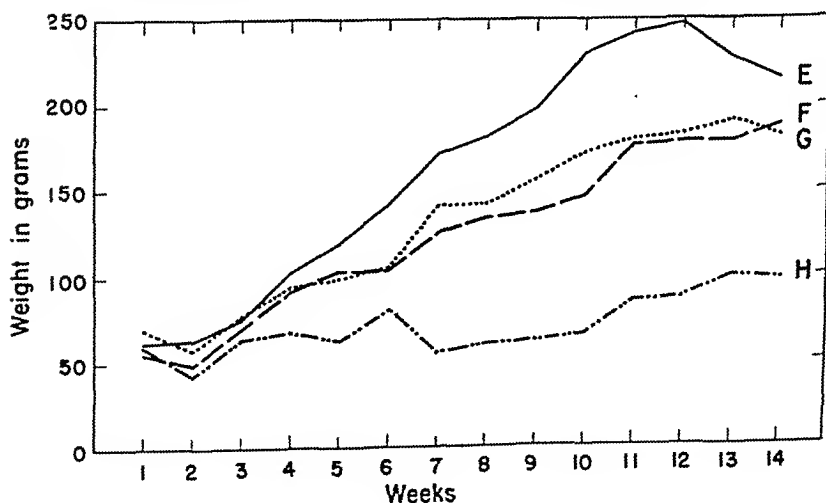


Fig. 2.

Gross Examination.—The tissues appeared grossly normal at autopsy.

Microscopic Findings.—*Liver:* Sections showed a very slight fatty metamorphosis uniformly scattered throughout the liver. *Kidneys:* Sections showed

the glomeruli to be normal. A few collections of lymphocytes were seen about a few of them. A few of the cells of the convoluted tubules showed slight hydropic changes.

Comment.—The changes in the organs might possibly have been due to a medicament but, if so, we would have to consider them to be extremely minimal. Similar findings are often found in animals without explainable causes.

Diagnosis.—Mild fatty metamorphosis of the liver.

DISCUSSION

Yohimbine seems to be fairly well tolerated in varying concentrations until a 1:1,000 proportion is fed *ad libitum*. Only one rat of four on this concentration survived the fourteen-week feeding period. Since yohimbine solution was taken in such sparing quantities (5 c.c. per day), one surmises that (1) distaste for the bitter alkaloid may have been responsible for the relative dehydration and (2) the latter in turn may have accounted for the slow and rather meager gain in weight in the lone survival in Group II. This is consistent with the autopsy findings, since the kidney, liver, and all other organs seemed to be essentially normal. In other words, yohimbine, when fed in the concentrations here employed, does not seem to be significantly histopathologic in white rats.

Appearance of three normal litters from rats fed on concentrations of 1:40,000 and 1:54,000 suggests that the drug does not affect reproduction in these concentrations.

Yohimbine solutions ingested in concentrations of 1:5,000 and 1:10,000 exerted no obvious toxic effect. This observation suggests that clinical trial with comparable doses per os or with proportionately smaller amounts parenterally might be made. Extreme caution should be exercised however in giving such a potent vasodilator in man.

SUMMARY AND CONCLUSION

Yohimbine, fed *ad libitum* to rats in drinking water, seems to be nontoxic for over three months except when the concentration in the water is increased to 1:1,000. On higher dilutions growth and vigor are generally maintained at good levels and habits are normal. Autopsy findings are essentially normal.

REFERENCES

1. Sollmann, T.: A Manual of Pharmacology, ed. 6, Philadelphia, 1942, W. B. Saunders Co., p. 326.
2. Yonkman, F. F., and Young, A. G.: The Antisymphathicomimetic Effect of Ethyl Yohimbine on Salivation and Mydriasis, *J. Pharmacol. & Exper. Therap.* 63: 40, 1938.
3. Hamet, R.: On a New Instance of the Inversion of the Effect of Adrenaline, *Compt. rend. Acad. d. sc.* 180: 2074, 1925.

NUTRITIONAL MACROCYTIC ANEMIA IN PATIENTS WITH PELLAGRA OR DEFICIENCY OF THE VITAMIN B COMPLEX

CARL V. MOORE, M.D., R. VILTER, M.D., V. MINNICH, M.S., AND T. D. SPIES, M.D.

THE macrocytic anemia which occurs in some patients with pellagra or other vitamin B complex deficiency states has not been thoroughly studied. Very little definite information about its pathogenesis has been accumulated. No assays of the gastric contents for intrinsic factor or of the liver for antipernicious anemia factor have been reported. It has been determined that patients with pellagra without a significant degree of macrocytic anemia have intrinsic factor in their stomachs¹ and that the liver of a patient who died of uncomplicated pellagra before any treatment had been instituted contained the antipernicious anemia substance,² but these observations cannot be accepted as applying to subjects who have macrocytic anemia in addition. The suggestion has been made that the anemia may be caused by a dietary deficiency of Castle's extrinsic factor^{3, 4} and in this regard possibly be similar to the nutritional macrocytic anemia found in tropical^{5, 12} and, more rarely, temperate zones.^{13-18, 27} Because achlorhydria and diarrhea are common attendant manifestations, sharp clinical differentiation of the anemia from addisonian pernicious anemia and from nontropical sprue has been difficult.¹⁹⁻²²

The investigations reported in this communication were undertaken as a study of the pathogenesis of the macrocytic anemia of pellagra. They demonstrate that there is a dietary deficiency of extrinsic factor associated in many, but not all, instances with poor absorption from the intestinal tract. Inadequate production of intrinsic factor was probably a contributing influence. Niacin, thiamine, riboflavin, calcium pantothenate, pyridoxine, inositol, para-aminobenzoic acid, and choline had no therapeutic value in correcting the anemia, even though they were given in combination both orally and parenterally. Administration of highly purified liver extracts, however, caused prompt hematologic and clinical improvement. It is suggested that the anemia is apparently more frequent in those regions of the United States where vitamin B deficiencies are endemic than is commonly realized. Its clinical manifestations are described and its relationship to sprue, tropical macrocytic anemia, and pernicious anemia are discussed. The cytologic characteristics of peripheral blood and bone marrow were found to be indistinguishable from those of addisonian pernicious anemia.

From the Department of Internal Medicine of the Washington University School of Medicine, St. Louis, Mo., and the University of Cincinnati, Cincinnati, Ohio. Studies in Nutrition at the Hillman, Ala.

The expenses of the general nutrition study have been borne by grants from a number of philanthropic persons, foundations, and commercial . . . which aid this study could not have been made. The expense of numerous patients in their homes and in the clinic over a long period of time has . . . nts from the Williams-Waterman Fund of the Research Corporation and . . .

Received for publication, Aug. 1, 1944.

1. MATERIAL AND METHODS

From 1940 to 1943, fifty-six patients with macrocytic anemia were observed in the Nutrition Clinic of the Hillman Hospital. From this group, twenty-five patients were selected for special study; their erythrocyte counts were under 3.0 million cells per cubic millimeter, and their diets had been grossly deficient in animal protein and the vitamins of the B complex for years. These subjects usually also had glossitis, pellagrous dermatitis, cheilosis, or peripheral neuritis. Complete medical and neurologic histories were always taken and physical and neurologic examinations performed. The dietary history was obtained and analyzed.* Red and white blood cell counts were done on peripheral blood with pipettes and counting chambers certified by the U. S. Bureau of Standards. Hemoglobin was determined as oxyhemoglobin with the Evelyn photocolormeter.²² Hematocrit determinations were made on oxalated venous blood (potassium oxalate, 4 mg., and ammonium oxalate, 6 mg. per 5 c.c. blood) centrifuged for thirty minutes in a Wintrobe tube at 3,000 r.p.m. Reticulocyte and platelet counts were made by the wet technique, using Dameshek's method.²⁴ Fixed reticulocyte preparations also were made each day.²⁵ Cover slip preparations for cytologic study of capillary blood and sternal marrow were stained both with Wright-Giemsa and supravital stains. The bone marrow specimens were obtained by sternal aspiration.

Gastric secretions were obtained for analysis both before and after histamine. The analyses were repeated every six to twelve months whenever achlorhydria was found on the first determination. Roentgenologic examination of the gastrointestinal tract was performed in each patient. Stool specimens were examined repeatedly for occult blood, ova, parasites, and pathogenic bacteria in all instances, and for fat and fatty acids in five patients.

Icteric indices were estimated in nine patients, and serum iron determinations were made in fourteen.²⁶ Intravenous and oral glucose tolerance curves were established for two subjects.

Detailed case summaries for all patients on whom data are presented in graphic form are given at the end of the paper.

2. CHARACTERISTICS OF THE ANEMIA

As in sprue and tropical macrocytic anemia, the anemia was cytologically indistinguishable from true Addisonian pernicious anemia. Red blood cells were macrocytic (mean corpuscular volume, 105 to 163 cubic microns) and either normochromic or slightly hypochromic (mean corpuscular hemoglobin concentration, 27 to 38 per cent) (Table I). They showed marked anisocytosis, poikilocytosis, and polychromatophilia (Fig. 1). An occasional nucleated red blood cell was found in the peripheral blood. Neutropenia and thrombocytopenia were characteristic findings. Nuclear hypersegmentation of both neutrophils and eosinophiles was the rule. Initial reticulocyte counts were usually low.

The sternal bone marrow was thick, red, and gelatinous; it contained only small amounts of fat. Microscopically, the marrow preparations appeared very

*The authors are indebted to Miss Jean Grant for her help in obtaining the diet histories and for analyzing them.

†By Miss Monette Springer and Mrs. Jane Mann, to whom the authors wish to acknowledge their indebtedness.

TABLE I

INITIAL EXAMINATION OF THE PERIPHERAL BLOOD AND GASTRIC JUICE ON TWENTY-FIVE PATIENTS WITH NUTRITIONAL MACROCYTIC ANEMIA

CASE	PATIENT	SEX	YEAR	R.B.C. (M)	Hb. (GM.)	RETICULO- CYTES (%)	W.B.C.	PLATELET	HEMAT.	M.C.V.	M.C.H.	M.C.H.C.	SERUM IRON (MG. %)	FREE ACID	COMBINED ACID	MAJOR SIGNS OF ASSOCIATED NUTRITIONAL DEFICIENCY DISEASES
1	I.R.	M	1940	1.56	7.5	1.1	2,800	96,400	25.5	163	48	29	.091	0**	3°	Pellagrous dermatitis; multiple neurtis; cheilosis
2	W.P.	M	1940	2.33	9.2	0.5	3,600	216,000	29.5	127	39	31	.051	30°	40°	Glossitis; multiple neuritis; chei- losis
3	E.A.	F	1940	1.62	7.2	6.2	3,350	49,000	23.0	142	44.5	31	.098	15°	20°	Glossitis; pellagrous dermatitis
4	J.S.	M	1940	1.56	6.5	1.9	2,850	-----	19.5	125	40	32	.119	26°	16°	Glossitis; pellagrous dermatitis;
5	E.P.	M	1940	2.22	9.3	0.6	4,450	109,600	29.0	130	42	32	.059	4°	14°	Glossitis; multiple neuritis; cheilosis
6	W.P.	M	1940	1.61	6.6	5.8	10,600	380,000	24.0	149	41	28	---	25°	12°	Pellagrous dermatitis; multiple neurtis
7	T.E.	M	1940	2.12	6.2	6.4	2,250	199,000	23.0	108	29	27	.242	17°	18°	Glossitis; pellagrous dermatitis;
8	M.P.	F	1940	1.52	4.9	0.4	2,500	392,000	17.0	112	32	29	.199	0**	22°	Glossitis; cheilosis
9	G.H.V.	M	1940	1.52	5.5	2.8	1,300	-----	16.0	105	36	34	.148	0**	12°	Glossitis
10	C.O.R.	M	1941	1.16	4.5	2.0	3,300	72,000	15.0	129	39	30	.153	52°	21°	Glossitis
11	T.F.D.	M	1941	1.47	4.9	7.6	3,100	148,000	16.0	109	32	31	.112	65°	11°	Multiple neuritis
12	M.S.	F	1941	1.98	7.8	0.6	4,600	554,000	26.0	131	40	30	.069	28°	25°	Glossitis; multiple neuritis
13	T.G.	M	1941	1.52	7.4	2.8	2,600	152,000	22.0	145	49	34	.132	51°	11°	Glossitis; multiple neuritis; chei- losis
14	W.H.	M	1941	1.57	7.0	1.0	4,750	-----	23.0	146	45	30	.172	0**	14°	Glossitis
15	B.W.	M	1942	1.3	5.2	1.0	3,300	-----	17.0	131	40	31	---	25°	13°	No diagnostic evidence of associ- ated deficiency disease
16	J.F.A.	M	1942	1.9	6.9	1.6	-----	-----	23.0	121	36	30	---	56°	16°	Multiple neuritis; cheilosis
17	E.O.G.	M	1942	2.21	7.9	1.0	4,300	153,000	27.5	124	36	29	---	82°	14°	Glossitis
18	O.B.	M	1942	1.2	5.3	0.6	4,250	240,000	16.0	133	44	33	---	16°	42°	Glossitis
19	G.S.	M	1942	1.54	7.1	2.2	6,500	-----	22.0	143	46	32	---	61°	17°	Glossitis; pellagrous dermatitis
20	N.T.	F	1942	1.70	5.7	0.6	4,050	-----	20.0	118	33.5	28.5	---	9°	19°	Glossitis; multiple neuritis; chei- losis
21	L.J.	M	1942	1.14	4.8	2.6	4,100	143,000	17.0	149	42	28	---	0**	12°	Pellagrous dermatitis; multiple neurtis
22	J.M.	M	1941	2.27	8.4	1.2	3,200	170,000	27.0	119	37	31	.101	0†	14°	Multiple neuritis
23	B.K.	F	1941	1.38	6.9	2.2	-----	-----	18.0	130	50	38	---	0†	10°	No diagnostic evidence of associ- ated nutritional deficiency dis- ease.
24	J.D.H.	M	1942	1.98	6.9	1.6	2,350	63,000	24.0	121	35	29	---	0†	20°	Pellagrous dermatitis; multiple neurtis.
25	J.R.	M	1943	1.70	8.2	0.6	8,400	-----	26.0	153	48	31.5	---	0†	24°	Glossitis; multiple neuritis

*Free hydrochloric acid found in gastric juice on subsequent tests. See Table VI.

†Free hydrochloric acid never found on subsequent tests. Diagnosis of nutritional macrocytic anemia still questionable. See Table VI.

cellular and the usual 2 to 1 or 3 to 1 predominance of myeloid over erythroid elements was altered in favor of an approximately equal distribution between the two forms of blood cell progenitors (Table II). There was, furthermore, a shift to the younger forms of nucleated erythroid cells with many megaloblasts and early erythroblasts. Megakaryocytes were present in either normal or slightly reduced numbers.

As will be emphasized later, the laboratory data obtained on these patients with macrocytic anemia differed in three ways from that usually found in pernicious anemia: (1) hydrochloric acid was usually present in the gastric juice; (2) the icterus index was within normal limits; and (3) the serum iron was usually not elevated.²⁷



Fig. 1.—Photomicrograph of peripheral blood film obtained from G. H. W. prior to therapy ($\times 1000$).

3. PATHOGENESIS OF THE ANEMIA

With few exceptions, patients were hospitalized whenever they were used for detailed study. They were permitted freedom of the wards if they felt well enough to be out of bed. Rigid control of their food intake was maintained at all times.

A. Evidence for an Extrinsic Factor Deficiency.—The diets of all patients included in this series had been deficient in animal protein for years. Any accurate estimation of the amount of extrinsic factor present in their diets was

TABLE II
DIFFERENTIAL BONE MARROW COUNTS ON TEN PATIENTS WITH NUTRITIONAL MACROCYTIC ANEMIA

CASE	PATIENT	YEAR	BASOPHILES	EOSINOPHILES (%)	MYELOCYTES (%)	RETICULOCYTES (%)	NEUTROPHILS (%)	BAND (%)	SEGMENTED (%)	LYMPHOCYTES	MONOCYTES	CLASMA TOCYTES (%)	PRIMITIVES (%)	MEGAKARYOCYTE AND PLASMA C	NOBLOASTS /100 W.B.C.	LATE ERYTHROBLASTS /100 W.B.C.	EARLY ERYTHROBLASTS /100 W.B.C.	MEGALOBLASTS /100 W.B.C.	NO. OF NUCLEATED R.B.C. PER 100 W.B.C.
4	J. S.	1940	0	1	C-45	13	1	11	0	0	0	0	17	M-1	8	52	34	44	108
7	T. E.	1940	0	4	B-2 C-61	9.5	3	5.5	2	0	0	0.5	13	P-3	18	32.5	25	21	96
8	M. P.	1940	0	3	B-0.5 C-66	12	6	8	0	0	0	1.5	2.5	M-1.0	38.5	32	29.5	16	116
9	G. H. W.	1941	0	1	C-41	9.5	3	7.0	0	0	0	8.0	29.5	M-0.5	71.5	28	25	22	146
10	C. O'R.	1941	0	2.5	C-53.5	15.5	5	16.5	0	0	0	0.5	6.0	P-0.5	29	10.5	18.5	21.5	79.5
12	M. S.	1941	0	2.5	C-75	7	0.5	3.5	0	0	0	3.0	8.0	M-0.5	39.5	20.5	10.5	12.5	83
13	T. G.	1941	0	1	C-33	14	7.5	10	0	0	0	33.5	33.5	-	8.5	25	12.5	21	67
14	W. H.	1941	0	3.5	C-47	12.5	5	14.5	0	0	0	0.5	16	M-1	59	21	18.5	24.5	123
18	O. B.	1943	0	5	C-30	9.5	16.5	30	2.5	0	0	0	5	-	17.5	6.5	20.5	21.5	64
22	J. M.	1941	0	4.5	B-1.0 A-0.5 C-43.5	9.5	2	10.5	0	0	0	0.5	28.5	P-1	25.5	27.5	10.0	16.5	99.5

impossible, however, because so few foods have been assayed for extrinsic factor activity. Known sources of the nutrient include beef muscle,²⁸ milk,²⁹ eggs,^{30, 31} liver,³² yeast,³³ and rice polishings and wheat germ.³⁴ All of these foods are also excellent sources of the vitamin B complex. Therefore, if it is true that extrinsic factor is usually associated in nature with the B vitamins, it is reasonable to assume that these patients, most of whom had clinical evidences of avitaminosis B, may also have had a deficiency of extrinsic factor.

TABLE III

	PROT. (GM.)	CAL.	CALCIUM (GM.)	IRON (MG.)	VIT. A (I. U.)	THI- AMINE (MG.)	RIBO- FLAVIN (MG.)	ASCORBIC ACID (MG.)
<i>Diet A</i>								
(Included hominy grits, biscuits, butter, salt pork, karo syrup, sugar, dried apples, black-eyed peas)								
Original diet	24	2319	.079	1.637	489	.5	.045	1.5
First revision	35	3010	.109	1.645	491	.8	.045	1.5
Second revision	37	3408	.116	2.205	491	.8	.045	1.5
<i>Diet B</i>								
1. No meat, fowl, or fish; no meat soups or meat gravies								
2. Not more than 1 egg and 2 glasses of milk daily								
3. As much as desired of all other foods								

In an attempt to study this possibility, five patients (Cases 1 to 5) with a macrocytic anemia of less than 2.5 million red blood cells per cubic millimeter were given 200 Gm. of ground beef muscle daily for a period of from seventeen to twenty-seven days. This amount of beef muscle was shown by Castle and his associates^{28, 35} to contain enough extrinsic factor to produce a reticulocyte response when added to 150 c.c. of normal human gastric juice and fed to patients with pernicious anemia in relapse. Four of these five subjects had free hydrochloric acid in the gastric juice; the fifth (Case 1) had a histamine refractory achlorhydria but was found one year later to have free acid when the analysis was repeated. As soon as hospitalization was instituted, the patients were given a very restricted diet calculated to be deficient in all the vitamins of the B complex (Diet A, Table III). A control period of a week or more preceded the period of beef muscle administration in each instance. The meat was fed either midway between breakfast and lunch or midway between lunch and the evening meal. A reticulocyte rise of from 5 to 15 per cent occurred in each subject; peak values were reached on the tenth to the fourteenth day. A characteristic response is illustrated in Fig. 2. In no instance, however, did the erythrocyte count show a significant rise. Observations were complicated by a number of factors. Glossitis was so severe in several patients as to make it difficult for them to take even fluids. To relieve these symptoms, it was necessary to give therapeutic amounts of niacin amide (J. S., Fig. 2). Pre-existing diarrhea was aggravated by the raw beef muscle, and the diet was so restricted that subjects objected to it vigorously. Furthermore, a moderately severe upper respiratory infection developed in each of the five patients during or immediately after the reticulocytosis. All of these factors may have combined to eliminate any erythrocyte increase that might otherwise have been obtained.

In order that some of these difficulties might be eliminated and the study be made more conclusive, a second series of observations was made. Six pa-

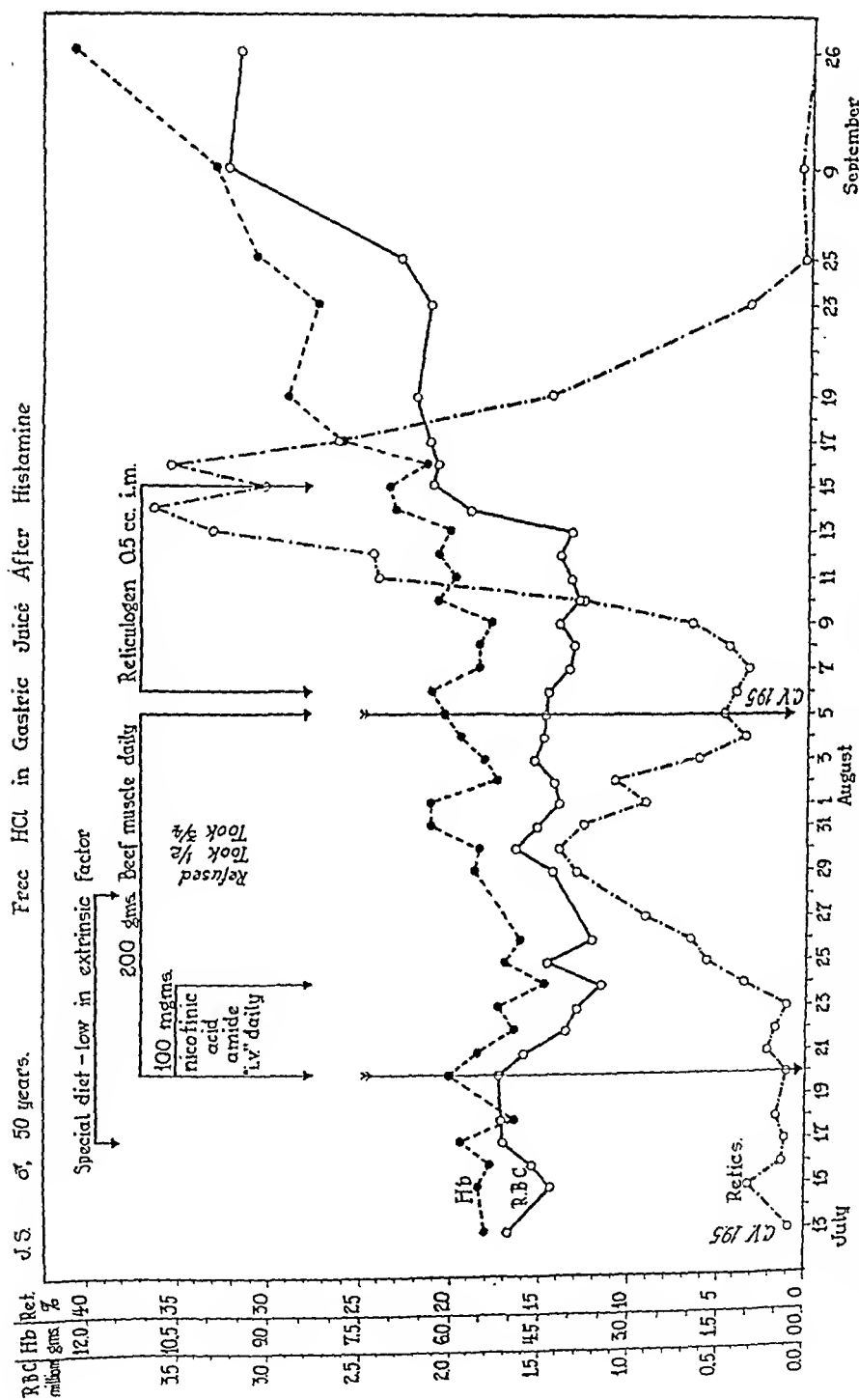


Fig. 2.—Submaximal hematologic response produced by daily administration of 200 Gm. of beef muscle to a patient with nutritional macrocytic anemia. Subsequent therapy with a highly concentrated liver extract injected intramuscularly caused a striking secondary reticulocyte response and rapid rise in the red blood cell count during a second period of study two years later, evidence was accumulated which suggested that absorption from the intestinal tract of this patient was impaired (see Fig. 6).

TABLE IV

COMPOSITION OF THE MIXTURE OF CRYSTALLINE B VITAMINS GIVEN DAILY FOR TEN DAYS TO SIX SUBJECTS (CASES 9 TO 14)

	ORALLY	INTRAVENOUSLY
Niacin (mg.)	200	50
Thiamine (mg.)	100	50
Riboflavin (mg.)	10	5
Calcium pantothenate (mg.)	20	10
Pyridoxine (mg.)	20	10
Inositol (mg.)	20	10
Para-aminobenzoic acid (mg.)	20	10
Choline (mg.)	20	10

tients were again selected (Cases 9 to 14). Four had free hydrochloric acid in the gastric secretions; the fifth and sixth had a histamine refractory achlorhydria but were later shown to have a return of free acid. A more liberal diet was permitted (Diet B, Table III), but it, too, contained no lean meat, liver, kidney, or cheese; one egg and two glasses of milk per day were permitted. During a control period of at least ten days, each subject was also given both orally and parenterally each day the amount of the crystalline B vitamins indicated in Table IV. Since no reticulocytosis developed, the control period served to establish the fact that none of the vitamins used possessed any extrinsic factor activity. This observation is in accord with that published recently by Castle and co-workers.³⁶ It also showed that the more liberal diet did not contain enough extrinsic factor to produce a reticulocyte response. Many of the associated clinical manifestations of avitaminosis were relieved by the vitamin therapy so that the subjects became more comfortable. The diarrhea was not entirely controlled by the vitamin administration, however, even though large doses of niacin or niacin amide were given during the whole period. Instead of using raw beef muscle, a fat-free 80 per cent alcoholic extract of beef was prepared according to the method of Formijne.³⁷ Enough extract was given between meals each day to be equivalent to 250 Gm. of beef. In several instances, twice this amount was fed for a portion of the time. The extract was more palatable than the raw beef muscle and caused less gastrointestinal discomfort. Reticulocytosis occurred in each case with peak values of from 7 to 31 per cent being reached on the seventh to the nineteenth day of therapy. There was concomitant subjective clinical improvement with increased appetite, a feeling of well-being, and lessened fatigue. Administration of beef extract was continued for as long as fifty days until an unequivocal rise of from 500,000 to 1,000,000 red cells per cubic millimeter had occurred (Fig. 3).

In several instances irregular rises in the reticulocytes developed so that two peaks were observed. The data obtained on C. O'R. (Fig. 4) illustrates this well. In this man an initial reticulocytosis of 19 per cent occurred. After fifteen days he was given the extract with meals in the hope that the gastric secretion stimulated at these times would cause more intrinsic factor to be produced and lead to a secondary response. A secondary reticulocyte rise to 31 per cent did occur, but it developed too rapidly to make it probable that the change in time of administration of the extract had anything to do with producing the result.

T. D. ♂. 65 years. 65° Free HCl after Histamine.

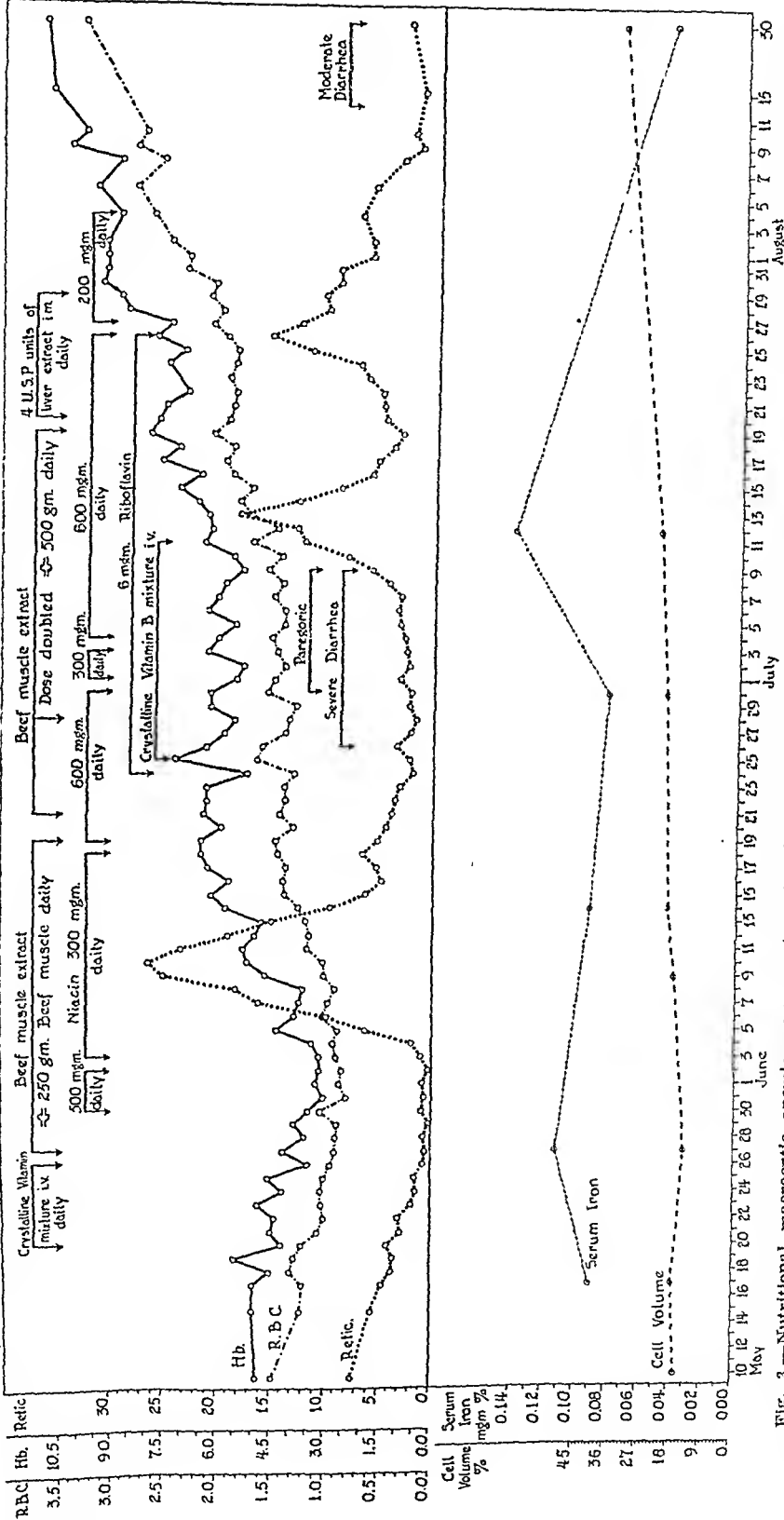


Fig. 3.—Nutritional macrocytic anemia—response to extrinsic factor and liver extract. The data illustrated in this chart demonstrate that the known crystalline B vitamins (B₁₂ and "folie acid" not included) are not capable of stimulating a hematologic response in patients with nutritional macrocytic anemia even when given both orally and parenterally. The response to a source of extrinsic factor suggests both that this factor was deficient in the diet and that intrinsic factor was present in the patient's gastric juice. Since a maximum therapeutic effect was not produced, however, until the amount of beef muscle extract was increased to one pound per day and the diarrhea was controlled, poor absorption from the intestinal tract probably also contributed to the pathogenesis of the anemia.

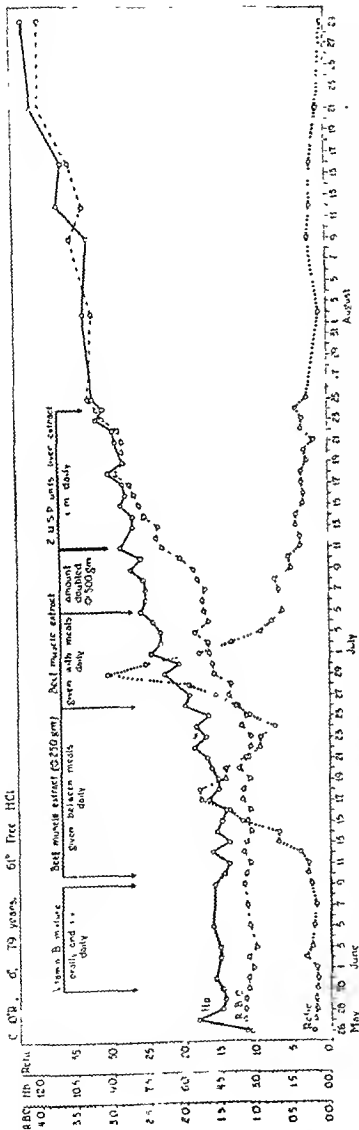


FIG. 1.—Nutritional macrocytic anemia—response to beef muscle extract. The data illustrated in this chart again demonstrate that the available crystalline vitamins of the B complex (biotin and "folic acid" not included) do not induce a hematologic response when given both orally and parenterally to a patient with nutritional macrocytic anemia. Satisfactory reticulocytosis and red blood cell increase occurred after the administration of an alcoholic extract of beef muscle as a source of extrinsic factor.

There seems little room for doubt that the beef muscle and its alcoholic extract contained some substance, presumably extrinsic factor, which induced a partial hematologic and clinical remission. Since beef muscle is an excellent source of the extrinsic factor, the conclusion seems justified that a deficiency of this substance probably existed in these patients.

B. Evidence of Poor Absorption From the Gastrointestinal Tract.—The presence of a persistent or intermittent diarrhea of long duration in most of the patients made it also appear likely that there was deficient absorption from the intestinal tract. Evidence to support this probability was more difficult to obtain. However, in nine of the ten subjects mentioned previously, daily injection of from 4 to 8 U.S.P. antipernicious anemia units in the form of highly purified liver extracts* (given after the response to beef muscle had occurred) produced additional reticulocyte elevations and, in eight patients, a marked acceleration in the rate of red blood cell rise (Figs. 2 and 3). This result admittedly could have been caused by poor absorption, by inadequate production of intrinsic factor by the patients, or by a combination of these two factors. Enough beef extract was supplied in the second series to make it seem likely that in these patients at least, an adequate supply of extrinsic factor was available.

Two additional observations make it appear probable that deficient absorption contributed to the suboptimal therapeutic results. T. G. (Fig. 5) developed a reticulocytosis of only 7 per cent following the daily ingestion of extract prepared from 500 Gm. of beef. A similar amount of extract was then added each day to 125 c.c. of normal human gastric juice and fed for fourteen days. A second small reticulocytosis occurred. During a third period, 1.5 units of oral liver extract* were given without any detectable effect, but when this was followed by the parenteral injection of liver extract, a third reticulocyte response was observed. In several other patients the oral administration of 2 units of liver extract† daily for fourteen days caused submaximal reticulocyte elevations so that secondary increases resulted when liver extract was subsequently given intramuscularly (Fig. 6). The parenteral doses were unfortunately larger than those which had been used orally, but the fact remains that the amounts given by mouth were great enough that they should have produced maximum hematologic responses. There is evidence, therefore, that deficient absorption may have contributed to the production of macrocytic anemia in some of the patients.

C. Intrinsic Factor Production.—The very fact that these individuals responded to the administration of beef muscle or its extract indicates that the gastric mucosa was elaborating enough intrinsic factor to produce at least a moderate amount of erythrocyte maturation factor. In addition, however, 125 c.c. of gastric juice were obtained daily for ten days from two of the subjects (Cases 3 and 11) and added to 200 Gm. of raw beef muscle in one case and to the extract from 250 Gm. of meat in the other. The mixture was fed in each instance to two patients with Addisonian pernicious anemia. Reticulocyte responses of 14.5 and 12.8 per cent, respectively, developed in the two test patients.

*Either as Reticulogen or as Purified Liver Extract, obtained from Eli Lilly and Co., containing 20 U.S.P. units per cubic centimeter.

†Hepovex, Eli Lilly and Co.

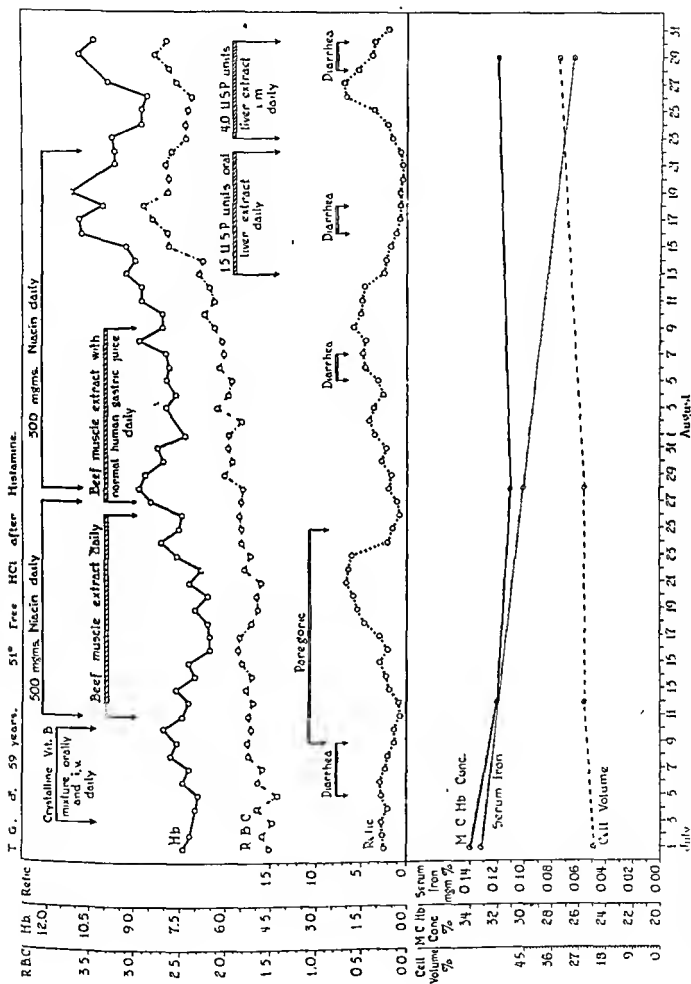
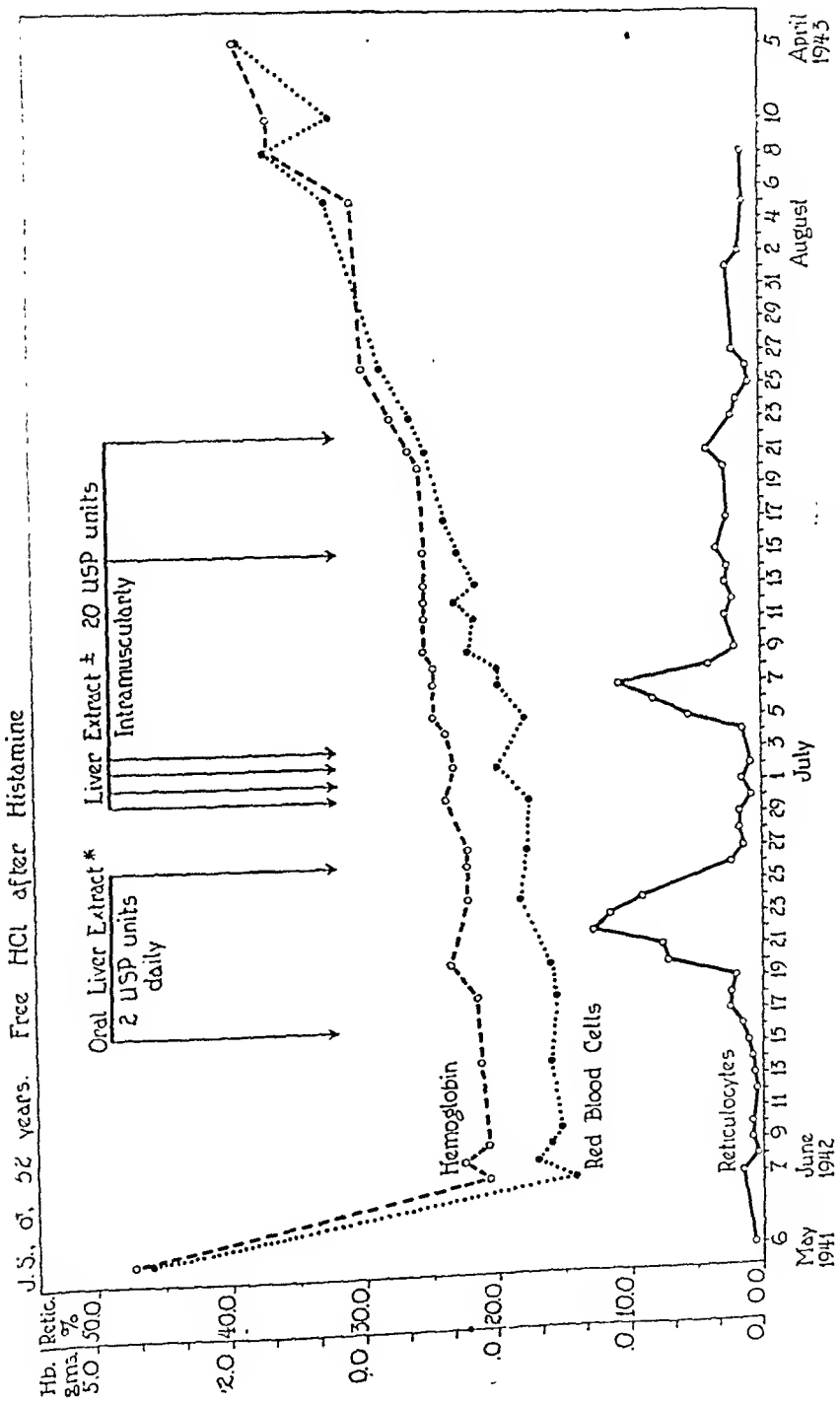


Fig. 5.—Nutritional macrocytic anemia—response to extrinsic factor and liver extract. In this patient there were submaximal reticulocyte responses to an alcoholic extract of beef muscle and to the extract plus 100 c.c. normal human gastric juice daily. A third rise did not occur when 15 U.S.P. units of liver extract were given orally each day but did follow the institution of parenteral liver therapy. It seems probable that dietary deficiency of extrinsic factor, poor production of intrinsic factor, and poor absorption from the intestinal tract all contributed to the development of the anemia.



* Hepavex (Lilly) ± Reticulogen (Lilly)

Fig. 6.—Response of a patient with nutritional macrocyte anemia to oral and parenteral liver therapy.

The presence of intrinsic factor in the gastric secretions of these two subjects, therefore, was proved by biologic assay.

As was emphasized in the previous section, the submaximal responses which followed administration of beef muscle or its extract may have resulted, in part, because the amounts of intrinsic factor elaborated by the gastric mucosa of these patients was less than normal. The actual production of erythrocyte maturation factor may have been inadequate, therefore, even when enough extrinsic factor was ingested. Castle and Rhoads were able to show that this was occasionally true in sprue.³⁹ The observations made on G. H. W. (Case 9) indicate that at least in some of these patients production of intrinsic factor was inadequate. This man developed a moderate reticulocyte response when he was given the extract from 250 Gm. of beef muscle daily; a second rise occurred after the dose was doubled (Fig. 7). During a third period the same amount of beef muscle was fed each day together with 100 cc of normal human gastric juice; a third reticulocyte elevation was obtained. This result indicates that his own stomach had not been producing enough intrinsic factor to utilize the extrinsic factor being provided to him. It is interesting that still a fourth reticulocyte response followed the parenteral administration of liver extract. In this man, then, three factors were probably participating in the pathogenesis of the anemia: (1) deficient intake of extrinsic factor; (2) inadequate production of intrinsic factor; and (3) poor absorption from the gastrointestinal tract.

D. Response to Highly Purified Liver Extracts.—It is important to emphasize that whenever the therapeutic response to oral therapy was suboptimal, the patients all responded with secondary reticulocyte rises when liver extract was given intramuscularly. The rate of red blood cell regeneration, furthermore, was almost always accelerated after injections of the extracts had been made. This observation is of interest since observers in India and Macedonia have reported that purified liver extracts (anahaemin^c) are not regularly effective in the treatment of patients with nutritional (tropical) macrocytic anemia,^{41, 42} while crude liver extracts given either orally or parenterally and autolyzed yeast fed by mouth produce excellent remissions.^{5, 6, 8, 9, 11, 12, 41} Wills⁴¹ has suggested that some factor of therapeutic importance is present in the crude extracts and autolyzed yeast in addition to extrinsic factor. She believed this second factor was lacking in the more highly purified liver extracts, like anahaemin, ordinarily used for the treatment of Addisonian pernicious anemia. More recently, however, other observers have found anahaemin therapeutically effective although large doses were required.^{40, 42-44} Since the patients studied in the present investigation responded quite satisfactorily to the injection of the most highly purified liver extracts then available, one must conclude either that (1) the nutritional macrocytic anemia found in the United States among patients with pellagra or other vitamin B complex deficiency states differs in some fundamental way from the nutritional macrocytic anemia seen in India, or that (2) the purified liver extracts used here contained the hypothetical substance not found by Wills in anahaemin. Available data are not sufficient to justify a more definite statement.

^cAnahaemin (British Drug House) is prepared by the method of Dakin and West and during preparation is completely precipitated with 99 per cent alcohol and taken through two ammonium sulfate fractionations and one reprecipitate. Two cubic centimeters of anahaemin contain the equivalent of 450 Gm. of original wet liver.⁴⁰

G. H. W., ♂, white. Histamine Refractory Achlorhydria.

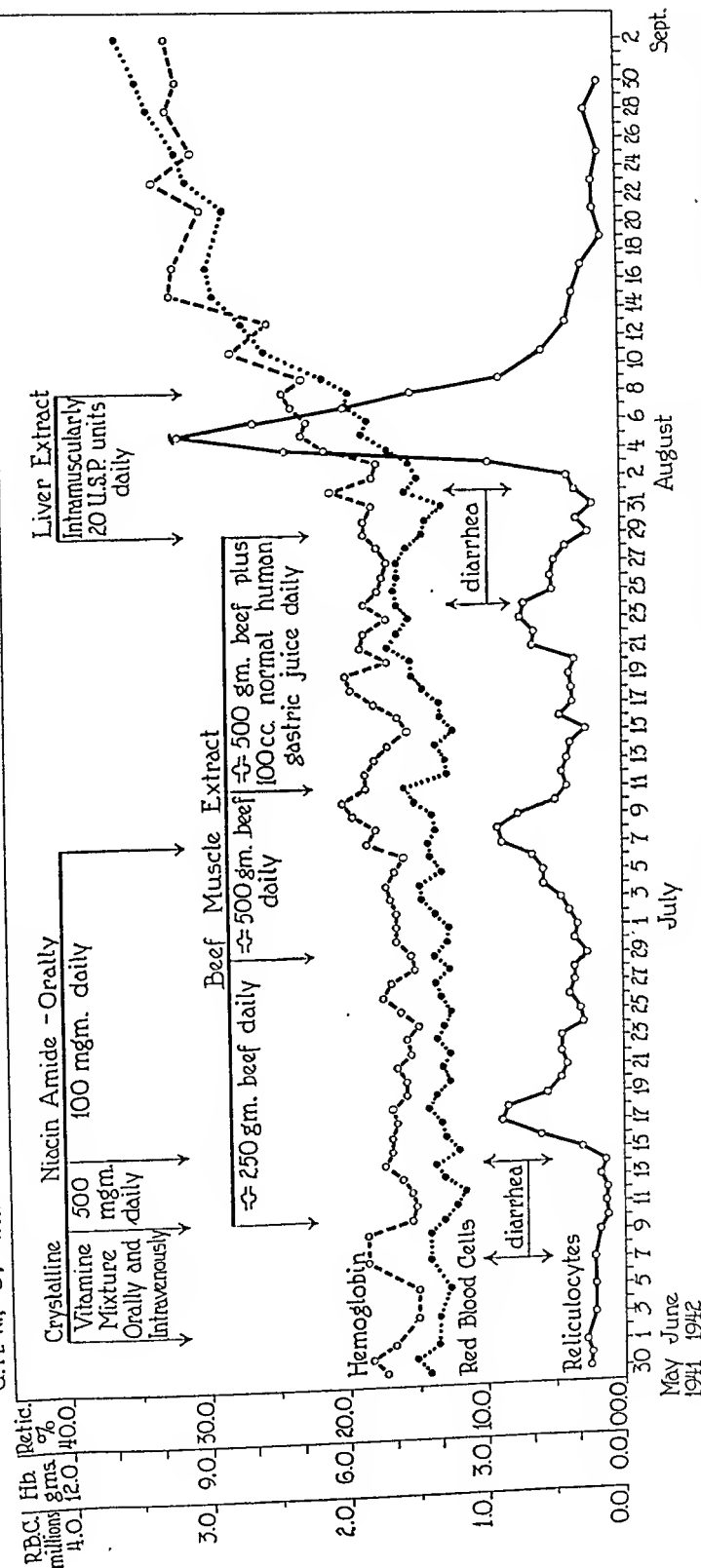


Fig. 7.—The charted data illustrate that this patient developed a submaximal reticulocyte response when given the equivalent of 250 Gm. of beef muscle daily (as an alcoholic extract). A second reticulocyte response occurred when the dose was doubled, and a third was observed when the beef extract was fed together each day with 100 e.e. of normal human gastric juice. These results suggest that there existed both a dietary deficiency of extrinsic factor and inadequate production of intrinsic factor by the patient's gastric mucosa. Since maximum hematologic improvement did not occur, however, until after liver extract was administered parenterally, poor absorption from the intestinal tract was probably also of pathogenic importance.

E. *Summary of the Studies on the Pathogenesis of the Anemia.*—The pathogenesis of this form of nutritional macrocytic anemia, therefore, seems to depend on the existence of a prolonged dietary deficiency of extrinsic factor associated in many instances with poor absorption from the intestinal tract and/or inadequate production of intrinsic factor by the gastric mucosa. Final proof cannot be obtained until pure extrinsic factor has been isolated and its effect tested on patients with similar anemia.

4. CLINICAL DESCRIPTION OF THE SYNDROME

A. *Clinical Manifestations.*—The ages of the patients with nutritional macrocytic anemia at the Nutrition Clinic of the Hillman Hospital varied from 34 to 80 years, but in the great majority of instances the age was greater than 50. The disease was observed only in white subjects and occurred three times as frequently in men as in women. Many of the men were bachelors or widowers. This fact suggests that their normally poor diets may have become even more deficient when the men were forced to cook for themselves. In all cases the diets had been grossly deficient in animal protein for years; one or two eggs a day or a glass of milk usually furnished the only animal protein consumed. No definite statement can be made about its incidence. The Nutrition Clinic of the Hillman Hospital draws patients for a radius of 100 miles. It cannot be a common disease in this region, therefore, but probably occurs more frequently than it is recognized. For reasons to be given later, it may readily be confused with addisonian pernicious anemia.

Clinical manifestations of the anemia per se could not be separated sharply from those caused by other deficiency states. In most cases, subcritical deficiencies of niacin, thiamine, or riboflavin had existed for many years with relapses occurring in spring and fall. Often the exacerbations of glossitis, dermatitis, epigastric distress, diarrhea, cheilosis, or peripheral neuritis had been associated with weakness, pallor, dizziness, tinnitus, and shortness of breath. This history suggested that the anemia may also have occurred previously. After many years of remissions and relapses, the weakness, dizziness, pallor, shortness of breath, and diarrhea became so distressing that the patients sought hospital aid. Physical manifestations were related chiefly to the skin, gastrointestinal tract, cardiovascular system, and the peripheral nerves.

Pellagrous dermatitis was present in eight patients and cheilosis as well as erosions of other mucocutaneous junctions in seven. In all patients the skin of the arms and legs was hyperpigmented, roughened, and dry. Irregular, coalescing lines of pigmentation following a vascular pattern (erythema ab igne of "fire burns") were common. The skin never had the fine texture and lemon tint seen in pernicious anemia.

All but two patients complained of persistent diarrhea for months before admission. The stools were loose, watery, and brown, although not unusually foul. Defecation caused no pain. Most bowel movements occurred shortly after meals. Four patients recalled brief episodes of thick, frothy, pale stools suggesting sprue, but such episodes never occurred while the patients were under hospital observation. Flatulence was present but was not disturbing; abdominal examination revealed mild distention. The liver and spleen were palpable only when complicating diseases such as malaria were present.

Painful burning sensations of the tongue, mouth, and epigastrium were distressing to all patients. These symptoms usually occurred when the diarrhea was severe, although two patients insisted that the onset of diarrhea relieved the burning sensations in the tongue. The tongue and oral mucous membranes varied in appearance from fiery redness and swelling to pallor and extreme papillary atrophy. No distinguishing features were found to differentiate these glossal changes from those of pellagra or pernicious anemia.

Cardiovascular abnormalities such as shortness of breath, paroxysmal dyspnea, and systolic murmurs were mild and seemed to depend only on the degree of anemia. All blood pressures were in the normal or low normal range. The pulse rate was not elevated until anemia became extreme and shock was impending. None of the patients showed the manifestations of "beriberi" heart.

Burning of the soles of the feet with cramping and soreness in the calf muscles were common complaints. Fifteen patients showed tenderness of the calves, stocking type of paresthesias, hypoaetive ankle jerks, and diminished vibration perception in the ankles, indicative of mild peripheral neuritis. In no case was definitive evidence for combined system disease discovered.

Oral temperatures above 99.6° F. never occurred unless infection was associated with the anemia. Severe weight loss was noted only when diarrhea had persisted for months. Peripheral edema, present in eleven patients, was always associated with a reduction in serum albumin.

B. Laboratory Data.—The hematologic characteristics of the anemia have already been defined. Gastric analysis revealed normal gastric acidity in fourteen patients, hypochlorhydria in two, and a histamine refractory achlorhydria in nine. Analyses were repeated at intervals of six months on the latter group of subjects. Free acid was found in the gastric secretions on at least one occasion in five of these patients; the remaining four patients consistently showed an achlorhydria (Tables I, VI, and VII).

The icterus index of all subjects on whom it was determined varied from 2 to 7. Serum iron values were either low or within the normal range (from 0.050 to 0.180 mg. per cent) in every instance but two (T. E., 0.242 mg. per cent; M. P., 199 mg. per cent). Glucose tolerance curves, done repeatedly by Dr. T. Maegruder on two patients, showed less than 20 mg. per cent increase after the

TABLE V
STOOL ANALYSES FOR FAT.

CASE	NAME	DESCRIPTION OF STOOL	TOTAL FAT/100 GM. MOIST FECES (GM.)	NEUTRAL FAT/ 100 GM. MOIST FECES (GM.)	FATTY ACIDS/ 100 GM. MOIST FECES* (GM.)
9	G. H. W.	Liquid, frothy, yellow-green	3.07		
11	T. T. D.	Soft, unformed, yellow, foul odor	1.37 2.38	0.76 1.34	0.61 1.04
12	M. S.	7/12, Fluid, brown 8/4, Soft, unformed	2.95 2.27	1.37 1.12	1.58 1.15
13	T. G.	Soft, light yellow	6.30	2.97	3.33
14	W. H.	Soft, yellow	1.97	0.99	0.98

*Calculated as stearic acid.

TABLE VI

COURSE OF PATIENTS WITH NUTRITIONAL MACROCYTIC ANEMIA AND 0° FREE ACID BEFORE TREATMENT
(IN EACH INSTANCE TREATMENT CONSISTED OF FROM 75 TO 150 U.S.P. UNITS OF CONCENTRATED LIVER EXTRACT)

CASE	PATIENT	1940				1941				1942				1943				1944			
		LOWEST		TREAT- MENT	HIGH- EST FREE ACID	LOWEST		TREAT- MENT	HIGH- EST FREE ACID	LOWEST		TREAT- MENT	HIGH- EST FREE ACID	LOWEST		TREAT- MENT	HIGH- EST FREE ACID	LOWEST		TREAT- MENT	HIGH- EST FREE ACID
1	L. R.	1.36	7.5	0°	0°	4.02	12.5	0°	0°	14.3	20°	0	0°	4.47	11.0	0°	0°	3.81	14.0	0°	14°
8	M. P.	1.53	4.9	0°	0°	3.65	9.6	0°	0°	1.42	4.3	0°	0°	1.02	4.0	0°	0°	3.81	12.5	0°	0°
9	G. H. V.	2.83	10.0	0°	0°	1.52	5.5	0°	0°	3.49	12.0	0°	0°	4.34	14.0	0°	0°	5.24	13.9	0°	0°
14	W. H.	3.42	10.0	-	-	1.57	7.0	0°	0°	1.74	6.4	0°	0°	Died							
21	L. J.					1.14	4.8	0°	0°	1.14	4.8	0°	0°	4.08	13.6	17°	0	5.22	15.3	-	0
<i>Course of Four Patients With Macrocytic Anemia and 0° Free Acid in Whom Diagnosis Is Still Questionable</i>																					
22	J. A.					2.27	8.4	0°	0°	4.70	-	0	0°	4.47	11.5	0°	0	3.95	12.4	0°	0°
23	B. K.					1.38	6.9	0°	0°	4.20	12.5	-	0	4.1	13.5	0°	0	3.65	12.1	0°	0°
24	J. D. H.					1.98	6.9	0°	0°			0°	0°	4.16	14.6	0°	0	4.58	14.0	0°	0°
25	T. R.									1.70	8.2	0°	0°	1.70	8.2	0°	0	3.04	12.4	0°	0°

0. No treatment.

Rx., Treatment with liver extract—from 75 to 150 U.S.P. units.

TABLE VII

COURSE OF PATIENTS WITH NUTRITIONAL MACROCYTIC ANEMIA WHO HAD FREE HYDROCHLORIC ACID BEFORE TREATMENT
(IN EACH INSTANCE TREATMENT CONSISTED OF FROM 75 TO 150 U.S.P. UNITS OF CONCENTRATED LIVER EXTRACT)

CASE-PATIENT	1940				1941				1942				1943				1944			
	LOWEST		HIGH-EST FREE ACID	TREAT- MENT	LOWEST		HIGH-EST FREE ACID	TREAT- MENT	LOWEST		HIGH-EST FREE ACID	TREAT- MENT	LOWEST		HIGH-EST FREE ACID	TREAT- MENT	LOWEST		HIGH-EST FREE ACID	TREAT- MENT
	R.B.C.	Hb.			R.B.C.	Hb.			R.B.C.	Hb.			R.B.C.	Hb.			R.B.C.	Hb.		
3 E. A.	1.62	7.2	15°	R	3.38	11.1	45°	0	3.55	11.5	Present	0	2.50	9.7	16°	0	-	-	-	-
4 J. S.	1.56	6.3	26°	R	4.12	14.8	48°	0	2.38	7.8	Present	R	3.87	11.7	Not det.	0	5.00	16.3	-	0
5 E. P.	1.96	8.2	4°	R	2.6	10.5	20°	0	4.0	14.0	Not det.	0	-	-	Not det.	0	4.87	15.0	-	0
6 W. P.					2.5	8.8	54°	0	3.75	13.2	Not det.	0	4.8	13.0	49°	0	3.80	13.0	-	0
11 T. I. D.					1.47	4.9	65°	R	3.8	12.0	Not det.	0	2.8	10.4	39°	R	-	-	-	-
12 M. S.					1.98	7.8	28°	R	4.39	13.6	Not det.	0	4.13	11.5	70°	0	4.88	14.0	62°	0
13 T. G. A.					1.52	7.4	51°	R	2.59	8.7	Not det.	0	4.44	12.2	68°	0	4.94	15.1	-	0
16 J. F. A.									1.90	6.9	Present	R	2.01	9.2	56°	0	3.75	12.0	-	0
17 E. O. G.									2.21	7.9	82°	R	4.1	14.5	52°	0	3.62	14.0	64°	0

0, No treatment.

Rx., Treatment with liver extract—from 75 to 150 U.S.P. units.

oral administration of glucose. Curves on the same patients were normal when glucose was given intravenously. Serum protein values for five patients (Cases 9 and 11 to 14) are recorded in Table VIII.

Repeated stool examinations for occult blood were negative in all patients in the series. Dr. Morriss Dexter demonstrated *Amoeba histolytica* in one patient, *Bacillus typhosus* in another, and *Shigella dysenteriae* in a third. Stool specimens from five patients were analyzed for free fat and fatty acids;^{45a} three of these subjects were selected from the four who gave a history of having had a spruelike diarrhea. In no case was either constituent found in excessive amounts (Table V). Values are recorded for moist feces rather than dry weight; they agree well with those reported for normal subjects.^{45b, c} Fluoroscopic examination of the gastrointestinal tract showed no abnormalities except for moderate dilatation of the colon in several instances; small intestinal patterns did not show the changes described in sprue.^{46, 47}

TABLE VIII
SERUM PROTEIN VALUES

CASE	PATIENT	DATE	TOTAL SERUM PROTEIN (GM. PER 100 C.C.)	ALBUMIN (GM. PER 100 C.C.)	GLOBULIN (GM. PER 100 C.C.)
9	G. H. W.	8/11/41	5.17	2.52	2.65
11	T. T. D.	7/17/41	4.74	3.72	1.89
		5/20/41	4.84		
		5/23/41	4.98		
		8/ 5/41	5.61		
12	M. S.	5/26/41	6.34	3.54	2.11
		5/30/41	5.42		
		8/ 5/41	5.65		
13	T. G.	8/11/41	5.41	3.58	1.83
14	W. H.	5/ 9/41	6.77	2.18	3.56
		5/20/41	5.69		
		5/29/41	5.66		
		8/11/41	5.74		

C. *Clinical Course*.—Coincident with the development of a hematologic remission, induced either by beef muscle or liver extract, the patients showed subjective improvement. Their appetites improved, they regained their strength, and they were able to resume their normal activities. After liver extract had been given, the diarrhea stopped. In the majority of instances, patients felt better and were more energetic than they had been for years. Two patients died, one from a myocardial infarct and the second from pulmonary tuberculosis. Pertinent data for all subjects who were followed for two or more years are recorded in Tables VI and VII. Seven of the twenty-five patients corrected their faulty dietary habits and began eating normal amounts of animal protein. They have remained well and free of diarrhea, and the anemia has not reappeared during the two or three years which have since elapsed, even though no liver therapy has been given during this time. They were not given special low fat diets. The other patients who returned to their inadequate food habits developed recurrences of the diarrhea, anemia, and other manifestations after eighteen to twenty-four months; they required further therapy.

5. RELATIONSHIP TO ADDISONIAN PERNICIOUS ANEMIA, SPRUE, AND TROPICAL MACROCYTIC ANEMIA

A. Addisonian Pernicious Anemia.—Statement has already been made of the fact that the nutritional form of macrocytic anemia described in this paper was cytologically indistinguishable from addisonian pernicious anemia. The chief points of differentiation were these: (1) twenty-one of the twenty-five subjects had free hydrochloric acid in the gastric secretions at least part of the time; (2) evidence already presented indicated that they also produced intrinsic factor; (3) the icteric indices were uniformly within normal limits; (4) with two exceptions, serum iron values were low or normal rather than high; and (5) each of the twenty-five subjects listed in Table I, to whom a source of extrinsic factor was given (Cases 1 to 5 and 9 to 14), responded with a reticulocyte response and usually with a red blood cell increase.* The presence of free hydrochloric acid in the gastric secretions of patients who apparently have true addisonian pernicious anemia rarely occurs,⁵⁰⁻⁵⁶ and in a few instances liver therapy is followed by a return of free acid in the stomach contents of subjects who initially had achylia gastrica.⁵⁷⁻⁵⁹ It is inconceivable that twenty-one of the twenty-five subjects here described could fit into this category.

Confusion with addisonian pernicious anemia could easily occur, however, at those times when an achlorhydria was present. The conditions of some of the patients (Cases 1, 8, 9, 14, and 21) might have been diagnosed as true pernicious anemia if response to extrinsic factor had not been tested, or if the patients had not subsequently showed free hydrochloric acid in the gastric secretions. Four subjects (Cases 22, 23, 24, and 25, end of Table I and Table VI), furthermore, never were observed to have free acid in the stomach contents, and, because they were used for other observations, their ability to respond to a source of extrinsic factor alone was not demonstrated. Their diets had been inadequate, their icteric indices were normal, and they presented the same clinical manifestations as did the remaining twenty-one subjects whose cases are recorded in Table I. It is entirely possible that they, too, had nutritional macrocytic anemia, but the differentiation of their anemia from pernicious anemia cannot be made on the basis of available data.

Since confusion of nutritional macrocytic anemia with pernicious anemia is likely to exist unless free gastric acid is present or response to extrinsic factor is demonstrated, it is entirely possible, in those regions where deficiencies of the vitamin B complex are endemic, that patients with nutritional macrocytic anemia are not infrequently diagnosed as having pernicious anemia. Some of the cases reported in the literature as examples of pernicious anemia with normal gastric acidity may actually be patients with nutritional macrocytic anemia. The practical importance of making the differentiation is evident when one con-

*The validity of using the response to extrinsic factor as a point of differentiation from addisonian pernicious anemia may be questioned since Wintrobe showed that remissions could be induced in patients with pernicious anemia by feeding 1 to 2 Gm. of brewers' yeast per kilogram per day.⁴⁸ Similar observations were made by Unzley and James.⁴⁹ However, only 12 Gm. of brewers' yeast are roughly equivalent to 200 Gm. of beef muscle in ability to form erythrocyte maturation factor when incubated with normal human gastric juice. The amounts of extrinsic factor contained in the large doses of brewers' yeast, therefore, are many times as great as those fed to the subjects in these observations. If the correct explanation of Wintrobe's results is that he provided tremendous amounts of extrinsic factor to interact with the small amount of intrinsic factor still produced by patients with pernicious anemia, then his observations are not quantitatively comparable to those reported here since the amounts of extrinsic factor given in these studies were not excessive.

siders that extrinsic factor deficiencies can be corrected by dietary means alone and do not require life-long administration of liver extract.

The animal experiments of Miller and Rhoads⁶⁰ suggest that dietary deficiencies maintained over a period of years might be an etiological factor in the development of pernicious anemia. There was no evidence, however, to indicate that the patients with continued poor diets tended to develop true pernicious anemia during the four years encompassed by this investigation; free gastric acid was sometimes first demonstrated during the second or third year of observation. These patients were almost certainly producing intrinsic factor in the first year of study. It is reasonable to assume that they maintained its production since several recovered the ability to secrete acid from two to three years later.

B. *Sprue*.—The relationship between the nutritional macrocytic anemia of pellagra and sprue is very difficult to grasp; differentiation may actually not be justifiable. The macrocytic anemia of sprue is apparently caused by poor intake of extrinsic factor, poor absorption of erythrocyte maturation factor, inadequate production of intrinsic factor, or a combination of these influences.^{38, 39} Its pathogenesis, therefore, may be similar to that described in the present study for nutritional macrocytic anemia. The anemia and bone marrow showed similar cytologic changes to those described for sprue.^{61, 62} Glossitis, flatulence, a persistent or intermittent diarrhea, a flat glucose tolerance curve, and other manifestations are common to both conditions. How much emphasis may be placed on the character of the diarrhea in differentiating between sprue and the macrocytic anemia of pellagra is problematic. The stools did, however, differ in character from those usually found in sprue. Only four of the patients included in this study gave a history of having large, frothy, fatty, foul stools. Moreover, even these four subjects failed to pass a spruelike stool during the time they were under observation. The fecal specimens, rather, were watery in type, not particularly foul. Hanes believes that the diagnosis of sprue cannot be made in the absence of steatorrhea.⁶³ The few analyses for fat and fatty acids which were made revealed no excessively high values. All of these patients responded satisfactorily to therapy with extrinsic factor or liver extract; none were refractory to treatment as is an occasional patient with sprue.^{63, 64} The seven patients, furthermore, who changed their intakes so as to include adequate amounts of animal protein have had no recurrence of either diarrhea or anemia. The fat in the diet was not controlled. Three or four years of observation are probably not enough to eliminate the possibility of a relapse, but it would be unusual to have seven patients with sprue all develop such long remissions at the same time.⁶⁴ The final answer to the question as to whether the two conditions represent two distinct entities or are identical may have to wait until their etiology is more completely understood.

C. *Tropical Macrocytic Anemia*.—Comparison of the syndrome described in this report with the nutritional macrocytic anemia of the tropics is difficult for two reasons: (1) clinical description of patients with the latter disease is not uniform; and (2) confusion exists as to the response they show to parenteral injection of purified liver extracts.

Nutritional macrocytic anemia in the tropics is apparently more common among women and appears quite frequently in association with pregnancy.^{5, 6, 42}

The patients are usually young or middle-aged adults who complain of shortness of breath, weakness, sore tongue, dysphagia, diarrhea and edema. About one-third show fever and splenomegaly; purpura may develop and uterine hemorrhage is common.¹¹ Paresthesias may be present but combined system disease has never been found. The anemia and bone marrow usually resemble or are identical with Addisonian pernicious anemia. Neutropenia may be severe. Achlorhydria is no more common than in the population at large. Bilirubinemia and urobilinuria were not observed by Wills,¹¹ but other observers have frequently found evidences of increased blood destruction.^{6, 8, 11} It is possible that the clinical picture of the disease varies in different regions or that the concomitant presence of other tropical diseases alters the clinical manifestations. In contrast, however, nutritional macrocytic anemia in Birmingham occurred three times as frequently in men as in women. Most of the patients had passed the age of 50 years. None of the women were pregnant. Splenomegaly was found only in those subjects who had previously had malaria. Evidences of increased hemolysis or of combined system disease were not observed. Signs and symptoms of pellagra or of other vitamin B complex deficiency states were common. These clinical differences between the two conditions may be fundamental, but they may also be produced in large part by differences in dietary habits and in the types of infectious and parasitic diseases to which patients are exposed.

When Wills demonstrated that marmite or yeast extracts produce brilliant therapeutic results in subjects with nutritional (tropical) macrocytic anemia,⁵ and when autolysed yeast was shown to be an excellent source of extrinsic factor,³³ it appeared probable that the anemia was caused by a dietary lack of extrinsic factor. This explanation was soon denied by Wills when she found that parenteral injection of purified liver extract (anahaemin) did not possess the therapeutic effectiveness of marmite or of crude liver extracts.⁴¹ She concluded that tropical macrocytic anemia is "due to a deficiency in the diet of some factor at present unidentified but other than Castle's extrinsic factor." The correctness of this suggestion has more recently been questioned again because other clinical investigators have found that anahaemin given parenterally was usually effective in the treatment of the disease.⁴²⁻⁴⁴ The response is apparently irregular. Some patients respond to moderate or small amounts, while others may show little improvement even though the dosage is large. The experience of Foy and Kondi in Macedonia adds even more confusion.⁴⁰ Anahaemin was effective when given to their female patients, but none of the males "respond to intramuscular liver therapy, no matter what preparation is used." These therapeutic observations are extremely important since they form the only clue to the etiology of the disease. Our patients with nutritional macrocytic anemia responded to the oral administration of beef muscle, beef muscle extract, or crude liver extract and to the parenteral injection of highly purified liver extracts. The evidence in favor of their having a deficiency of extrinsic factor, therefore, is as good as can be obtained until pure extrinsic factor has been isolated. Furthermore, final decision as to the possible etiologic identity of tropical macrocytic anemia and the form of nutritional macrocytic anemia described here will probably have to wait until pure extrinsic factor is available.

6. SUMMARY

The occurrence of macrocytic anemia is described in fifty-six patients who had existed for years on diets inadequate in animal protein and in the vitamins of the B complex. Most of these subjects also had clinical evidence of pellagra, ariboflavinosis, or beriberi. In twenty-five, the red blood cell count ranged from 1 to 3 million cells. Males predominated in a ratio of 3 to 1, and most of the patients were older than 50 years of age. The most striking clinical manifestations were weakness, pallor, glossitis, and intermittent or persistent diarrhea. The skin either showed the presence of pellagrous dermatitis or was rough, hyperpigmented, and dry. Splenomegaly was found only in those patients who had previously had malaria. Eighteen subjects showed signs of mild peripheral neuritis, but combined system disease was not observed. Physical or laboratory evidences of increased hemolysis were absent. The peripheral blood and bone marrow were cytologically indistinguishable from those of Addisonian pernicious anemia. Free hydrochloric acid was found in the gastric contents of sixteen patients at the time they were first studied; in five others, free acid was found on subsequent examinations.

All of the subjects showed prompt therapeutic response to the parenteral injection of highly purified liver extracts. In addition, it was demonstrated that they would develop reticulocyte rises when fed beef muscle, an 80 per cent alcoholic extract of beef muscle, or crude liver extract. When the oral administration was prolonged, the red blood cells also increased in number. Studies of the pathogenesis of the anemia indicate that it is probably caused by a dietary deficiency of extrinsic factor associated in many, but not all, instances with poor absorption from the intestinal tract. Inadequate production of intrinsic factor is probably also a contributing influence. Thiamine, niacin, riboflavin, calcium pantothenate, pyridoxine, inositol, para-aminobenzoic acid, and choline given together both orally and parenterally did not affect the erythropoietic equilibrium.

The similarity, points of difference, and possible relationship of this form of nutritional macrocytic anemia to sprue and to tropical macrocytic anemia are discussed. Emphasis is given to the fact that nutritional macrocytic anemia in patients who have an intermittent achlorhydria may easily be confused with Addisonian pernicious anemia. It is suggested that this syndrome may be more common in those regions of the United States where pellagra is endemic than has formerly been realized.

CASE REPORTS

J. S., a white man 50 years of age, had had diarrhea alternating with constipation from 1937 until admission to the hospital July 16, 1940. He described the diarrheal stools as being watery and brown; they occurred from ten to twelve times a day. The attacks of diarrhea were accompanied by sore tongue and mouth, burning in the epigastrium, and burning sensations in the feet. During the month before admission he had become weak and short of breath on exertion.

His diet consisted chiefly of dried beans or peas, white bread or corn biscuits, and occasionally eggs, tomatoes, and fish.

Physical examination showed a well-developed, moderately nourished white man. His hair was gray and his tongue was smooth and swollen, but not red. The oral mucous membranes were pale. Examination of the heart, lungs, and abdomen revealed no abnormalities.

Blood pressure was 128/80. The skin of the arms and legs was hyperpigmented, especially over the elbows and knees. Neurologic examination was normal except for tenderness of the calves and feet.

Kahn reaction was negative. Urinalysis revealed no abnormality. Stool examination was consistently negative for occult blood, pathogenic bacteria, and parasites. Examination for microscopic fat was negative. The gastric juice contained 26° free hydrochloric acid and 42° total acid 20 minutes after histamine stimulation. Fluoroscopic examination of the gastrointestinal tract showed no abnormalities except for spasticity in the cecum and colon. For hematologic data see Tables I and II. Bone marrow examination showed many megaloblasts and early erythroblasts.

The patient was fed Diet A (see Table III) and given paregoric and niacin to control the diarrhea. The oral temperature varied between 98.6 and 100° F. After a control period of ten days, he was fed 200 Gm. of raw beef muscle daily. The reticulocytes reached a maximum of 14 per cent on the sixth day of beef muscle therapy (see Fig. 2), but there was no detectable increase in the red blood cells after twelve days of therapy. During the last seven days of this period, however, he suffered from a severe upper respiratory infection. One-half cubic centimeter of reticulogen (approximately 10 U.S.P. units) was given intramuscularly daily for ten days. The reticulocytes reached a maximum of 34.5 per cent on the eleventh day and the red blood cell count rose rapidly.

In 1941, the patient was observed in the clinic on several occasions. He continued to subsist on the inadequate diet but had no anemia. A gastric analysis showed 48° free hydrochloric acid and 61° total acid after histamine. He developed no clinical evidence of nutritional deficiency disease and received no specific treatment during that year.

In the spring of 1942 he returned to the clinic with dermatitis, diarrhea, and weakness. His tongue was bright red, swollen, and painful, and there was a sealy, hyperpigmented, and symmetrical pallagrous dermatitis on the ankles. The red blood count had fallen to 2.38 million per cubic millimeter, and the hemoglobin to 7.8 Gm.; the mean corpuscular volume was 106 cubic microns. He was admitted to the hospital and fed Diet B (see Table III). After a control period of ten days, he was given a liver extract orally in doses equivalent to 2 U.S.P. units per day. A reticulocyte maximum of 12.8 per cent was reached on the seventh day of therapy and the red blood cells increased slowly. This submaximal response to oral liver therapy was followed by another reticulocyte response and a rapid red blood cell rise when 20 U.S.P. units of liver extract were given intramuscularly for three days (Fig. 6).

The patient returned to the clinic in the spring of 1943 and 1944. He had a mild normocytic normochromic anemia on each occasion (red blood cell count, 3,870,000; hemoglobin, 11.7 Gm.), but felt well and was working regularly as a farmer.

T. T. D., a white male 63 years of age, was admitted to the Hillman Hospital May 12, 1941, with the chief complaints of diarrhea, weakness, burning of the tongue, and dependent edema, all of from five to eight months' duration. He gave a history of intermittent diarrhea since 1917. The stools during these episodes were usually thin, watery, and brown; occasionally, however, they were bulky, creamlike, and foul. The interval between attacks was often measured in years. In 1919, when the diarrhea was so severe that he defecated from twenty to thirty times a day, he developed a psychosis from which he recovered after receiving some type of parenteral medication and being fed an "egg diet." His next incapacitating illness occurred in 1938 when the diarrhea was accompanied by periods of mental confusion, burning sensations of the tongue and abdomen, and dermatitis of the hands, feet, and neck. This time the patient "cured" himself by eating a dozen raw eggs daily. He was able to resume his work as a house-to-house salesman and remained well until August, 1940, when diarrhea developed again. During the following months, he developed the symptoms described by the chief complaints mentioned above.

His diet as reported by him consisted of lean meat, eggs, milk, sweet and Irish potatoes, green vegetables, fruits, cornbread, biscuits, and puddings. It is doubtful, however, if the dietary history represented his usual fare since he was an itinerant picture frame salesman who traveled over much of Alabama visiting and eating in the homes of many Nutrition Clinic patients who were known to have deficient diets.

He was a thin, pasty-appearing man who weighed 138 pounds. His tongue and oral mucous membranes were pale, swollen, and smooth. There were scars at both angles of the mouth and the skin of the face, arms, and legs was yellowish brown. There were several discrete retinal hemorrhages. The heart was moderately enlarged, but no murmurs were heard; blood pressure, 92/58. The lungs and abdomen were within normal limits. The ankles showed pitting edema. Neurologic examination revealed mild "sock" type of hyperesthesia over the feet up to the ankles, absent vibration perception in the lower extremities, and absent ankle jerks.

Blood Kahn reaction was negative. Urine examination was normal. Stool examination was repeatedly negative for occult blood and parasites, but on one occasion an atypical shigella organism was found. Values for the fat content of the stool are recorded in Table V. X-ray examination of the barium-filled colon revealed moderate atony and dilatation. Fluoroscopic examination of the upper intestinal tract showed only a moderate hypermotility. Gastric juice following histamine stimulation contained 65° free acid and 76° total acid. Hematologic data are summarized in Tables I and II.

He received the vitamin mixture orally and parenterally for eight days with improvement of the burning sensations in the tongue but without any reticulocyte response (Fig. 3). The red cell count and hemoglobin decreased steadily. During this period, 100 c.c. of gastric juice were withdrawn before breakfast each morning, pooled, and fed with beef muscle to a patient with pernicious anemia in relapse. He was given orally an amount of alcoholic extract of beef muscle equivalent to 250 Gm. of beef per day for twenty-three days. The reticulocytes rose to 25.2 per cent on the fifteenth day, and there was a gradual increase in the red cell count and hemoglobin. After an interval of three days the beef extract was given again and was doubled in amount when, despite daily oral therapy with niacin, the patient's diarrhea and glossitis increased in severity. This attack was controlled with paregoric given orally and niacin given intravenously. There was a secondary reticulocyte rise to a maximum of 23.8 per cent on the sixteenth day after the dose of beef extract had been doubled and on the seventh day after the diarrhea had stopped.

The patient remained well and able to work during 1942. During the spring of 1943 he noted slight loss of strength but no diarrhea or sore tongue. The red blood count had fallen to 2,800,000 and the hemoglobin to 10.4 Gm.

C. O'R., an 80-year-old cantankerous white man, entered the clinic May 31, 1941, because of weakness and sore tongue for six months. These symptoms were gradual in onset and were associated with loss of appetite and constipation. For the past eight years his diet had consisted of pancakes, corn flakes, rice mush, potatoes, a few eggs, and canned milk. He denied previous serious illness or the occurrence of pellagra in any member of his family.

He was a pale, emaciated old man with bilateral lenticular cataracts. His tongue and oral mucous membranes were pale, smooth, tender, and spotted with a necrotic grayish membrane which would not wipe off. His skin was dirty, and on the arms and legs there were small, discrete, indurated ulcers. No abnormalities were found on examination of the chest, heart, and abdomen. Blood pressure was 117/75. He had mild edema of the feet and ankles. The neurologic examination revealed absent ankle jerks and absent vibration perception in the lower extremities.

The patient weighed 115 pounds, the Kahn reaction was 3 plus, and the urine revealed no abnormality. Stool examinations were persistently negative for occult blood, pathogenic bacteria, and parasites. Gastric juice contained 52° free acid after histamine stimulation. X-ray examination of the barium-filled colon showed general dilatation and redundancy. Fluoroscopic examination of the stomach and small intestines were within normal limits. Tables I and II summarize the data obtained on this patient's peripheral blood and bone marrow. He was fed Diet B (see Table III), and after a control period of five days, the mixture of crystalline vitamins was given orally and intravenously for eleven days. No reticulocyte response occurred but the gray membrane and the burning sensations in the tongue disappeared and the skin ulcerations healed. For the next thirty-one days the patient received an alcoholic extract of beef muscle in amounts as indicated in Fig. 4. Two definite reticulocyte peaks occurred: one of 16 per cent on the ninth day and the second of 35 per cent on the nineteenth day, five days after the routine was changed to the administration

of beef extract with meals. A significant increase in the number of red blood cells also occurred. No additional reticulocytosis developed when the dose of beef extract was doubled (on the twenty-fifth day) nor when 2 U.S.P. units of concentrated liver extract were given intramuscularly each day for ten days. The patient's appetite and disposition improved after the beef extract had been administered for a week. When he was discharged, he weighed 156 pounds and was greatly improved. Observations were terminated six months later when he died following a coronary occlusion.

T. G., a 59-year-old white man, had been bothered with diarrhea and sore tongue every summer for the past eight years. At the time of admission to the Nutrition Clinic in July, 1941, the stool consisted of bulky, whitish, soft material, sometimes foamy and foul smelling. He complained of headache, dizziness, ringing in the ears, blurring vision, watering of the eyes, photophobia, sore red tongue, and burning of the skin of the arms and legs. These last two symptoms had increased in severity since the first of April, 1941. He had pain and weakness in the hands and feet, and itching on the backs of the arms and hands. There had been a weight loss of six pounds in two months.

His diet consisted of milk, fat meat, dried vegetables and fruits, corn bread, biscuits, and coffee.

The patient was a moderately well-developed, poorly nourished white man. The conjunctivae were pale. There were a few atrophic areas in the right fundus and many in the left fundus. A large flame-shaped hemorrhage was visible along the inferior temporal artery. His hair was gray. The lips were mottled and showed transverse fissuring and atrophy. Fissures and sears with some maceration were present at each angle of the mouth. The tongue was swollen, extremely atrophic, and flecked with areas covered by a whitish membrane; many red, pin-point spots were visible along the tip and sides. Purpura was present on the hands, legs, and neck. Extensive brown pigmentation was present on the face, hands, arms, and feet. There was mild pitting edema of the ankles. Examination of the heart, lungs, and abdomen revealed no abnormality. Blood pressure was 118/70. Vibratory perception was normal in the hands and feet. The position of the toes was normally perceived. The calves of the legs and soles of the feet were tender to pressure, but no skin paresthesias were present.

Reflexes	Right	Left
Biceps	+++	+++
Triceps	+++	+++
Knee Jerks	++	++
Ankle Jerks	+	+
Babinski	Flexor response	Flexor response
Chaddock	Flexor response	Flexor response

Kalm reaction was negative. Urinalysis showed only a faint trace of albumin. The stool was soft to liquid in consistency, yellow, and occasionally contained a small amount of foam; it contained no blood or pathogenic bacteria. On one examination motile forms of *Endamoeba histolytica* were seen. Values for the fat content of the feces are recorded in Table V. Fluoroscopic examination of the gastrointestinal tract was normal except for atony of the colon. The gastric juice after histamine stimulation contained 51° free acid. Oral glucose tolerance test produced a low flat curve, while the intravenous curve was normal. Hematologic data are summarized in Tables I and II.

There was a gradual improvement of bowel function during the entire period of study (Fig. 5). The patient's strength improved, purpura and edema disappeared, and the tongue and oral mucous membranes regained their normal color though the papillae remained atrophic. Anemia was again present in 1942, but the red blood cell count was within normal limits in the summers of 1943 and 1944. Diarrhea and other symptoms did not return. No liver extract was given after 1941, and no precautions to control dietary fat were taken.

G. H. W., a 79-year-old white man, was admitted to the Nutrition Clinic May 31, 1941, because of weakness and soreness of the mouth which had gradually increased in severity during the preceding four years. Because of food idiosyncrasy he had limited his diet to white meal, canned tomatoes, spaghetti, and okra during this period of time. On admission he complained of mild headache, ringing in the ears, burning of the eyes, sore mouth, watery

diarrhea, aching in the legs, and numbness and tingling in the hands and feet. He admitted having had hallucinations but had good insight. His wife was under observation in the Nutrition Clinic because of pellagrous glossitis and cheilosis.

The patient was an emaciated white man, with pallid, sallow, extremely atrophic skin, swollen tongue, and buccal mucous membranes. The heart, lungs, and abdomen were within normal limits. The blood pressure was 115/80. Vibration perception was present in the arms but absent in the legs. There were no paresthesias or ataxia and the position of the toes was perceived accurately. The deep reflexes were equal and active. The Chaddock test caused dorsiflexion of the great toes bilaterally, but the rest of the plantar responses were normal.

The Kahn reaction was negative. Urinalysis showed no abnormality. Stool examination was negative for occult blood, pathogenic bacteria, and parasites. Stool analysis for fat showed 3.07 Gm. total fat per 100 Gm. of moist feces. There was no free hydrochloric acid in the gastric juice after histamine stimulation, but free acid was found on two occasions in 1942 and 1943. Fluoroscopic examination of the gastrointestinal tract revealed no abnormalities except hypermotility.

During the control period on the vitamin mixture (Fig. 7), the patient had much less diarrhea than formerly (a reduction in number of stools from ten to two or three per day), but the stools continued to be watery. The swelling and burning of the tongue disappeared, but the atrophy was unchanged. There was no change in the neurologic examination, and the patient continued to have periods of mental confusion. This situation continued unchanged until the patient was given intramuscular liver extract. Following liver therapy there was a rapid increase in strength and complete relief from the diarrhea. He was last seen during the summer of 1944 and had had no recurrence of his symptoms or of the anemia. He had received no liver extract since 1942.

REFERENCES

1. Spies, T. D., and Payne, W.: A Study of the Etiological Relationship Between Pellagra and Pernicious Anemia, *J. Clin. Investigation* 12: 229, 1933.
2. Sydenstricker, V. P., in Harris, S., and Harris, S., Jr.: Clinical Pellagra 16: 248, St. Louis, 1941. The C. V. Mosby Co.
3. Bianco, A., and Jolliffe, N.: The Anemia of Alcohol Addicts; Observations as to the Role of Liver Disease, Achlorhydria, Nutritional Factors and Alcohol on Its Production, *Am. J. M. Sc.* 196: 414, 1938.
4. Moore, C. V., Vilter, R., Minnich, V., and Spies, T. D.: Nutritional Macrocytic Hyperchromic Anemia, *J. A. M. A.* 118: 1161, 1942.
5. Wills, L.: Studies in Pernicious Anemia of Pregnancy. VI. Tropical Macrocytic Anemia as a Deficiency Disease, With Special Reference to the Vitamin B Complex, *Indian J. M. Research* 21: 669, 1934.
6. Napier, L. E.: Tropical Macrocytic Anemia, *Lancet* 2: 679, 1936.
7. Giglioli, G.: *Rep. Surg. Gen. of Brit. Guiana*, 1934, Appendix II B, p. 94.
8. Fairley, N. H., Bromfield, R. J., Foy, H., and Kondi, A.: Nutritional Macrocytic Anemia in Macedonia, *Tr. Roy. Soc. Trop. Med. & Hyg.* 32: 132, 1938.
9. Rodriguez-Molina, R.: Tropical Macrocytic Anemia in Puerto Rico, *Puerto Rico J. Pub. Health & Trop. Med.* 15: 177, 1939.
10. Anderson, T. F., and Roberts, J. I.: Macrocytic Anaemias in Kenya—Preliminary Report, *Tr. Roy. Soc. Trop. Med. & Hyg.* 33: 615, 1940.
11. Trowell, H. C.: Deficiency Anaemias of Malnutrition, *Lancet* 1: 43, 1943.
12. Sippe, G. R.: Autolysed Yeast for Nutritional Macrocytic Anaemia, *Brit. M. J.* 1: 656, 1944.
13. Kern, R. A.: Diet as a Factor in the Etiology of Anemia, *Ann. Int. Med.* 5: 729, 1931.
14. Barnett, C. W.: The Significance of the Gastric Secretions in Pernicious Anemia, *Am. J. M. Sc.* 182: 170, 1931.
15. Ungley, C. C.: The Effect of Yeast and Wheat Embryo in Anemias. I. Marmite, Yestamin, and Bemax in Megalocytic and Nutritional Hypochromic Anemias, *Quart. J. Med.* 2: 381, 1933.
16. Groen, J., and Snapper, I.: Dietary Deficiency as a Cause of Macrocytic Anemia, *Am. J. M. Sc.* 193: 633, 1937.
17. Ungley, C. C.: Some Deficiencies of Nutrition and Their Relation to Disease. II. Nutritional Deficiency in Relation to Anemia, *Lancet* 1: 925, 1938.
18. Alsted, G.: Exogenous Pernicious Anemia, *Am. J. M. Sc.* 197: 741, 1939.
19. Hines, E. A.: Confusion of Pellagra With Sprue, *J. South Carolina M. A.* 26: 336, 1930.

20. Alessandrini, P.: Relations Between Sprue, Achylia and Pellagra, Arch. ital. d. mal. d. app. diger. 2: 631, 1934.
21. Haden, R. L.: Multiple Specific Deficiency Disease in the Adult, J. A. M. A. 106: 261, 1936.
22. Harris, S., and Harris, S., Jr.: Clinical Pellagra, St. Louis, 1941, chap. 13, Tho C. V. Mosby Co., pp. 163-183.
23. Evelyn, K. A.: A Stabilized Photoelectric Colorimeter With Light Filters, J. Biol. Chem. 115: 63, 1936.
24. Dameshek, W.: Method for Simultaneous Enumeration of Blood Platelets and Reticulo-eytes With Consideration of Normal Blood Platelet Count in Men and in Women, Arch. Int. Med. 50: 579, 1932.
25. Wintrobe, M. M.: Clinical Hematology, Philadelphia, 1942, Lea & Febiger, p. 58.
26. Moore, Carl V., Minnich, V., and Welch, Jo: Studies in Iron Transportation and Metabolism. III. The Normal Fluctuations of Serum and "Easily Split-Off" Blood Iron in Individual Subjects, J. Clin. Investigation 18: 543, 1939.
27. Moore, Carl V., Doan, C. A., and Arrowsmith, W. R.: Studies in Iron Transportation and Metabolism. II. The Mechanism of Iron Transportation: Its Significance in Iron Utilization in Anemic States of Varied Etiology, J. Clin. Investigation 16: 627, 1937.
28. Castle, W. B., and Townsend, W. C.: Observations on Etiologic Relationship of Achylia Gastrica to Pernicious Anemia; The Effect of the Administration to Patients With Pernicious Anemia of Beef Muscle After the Incubation With Normal Human Gastric Juice, Am. J. M. Sc. 178: 764, 1929.
29. Miller, F. R., and Pritchard, W. H.: Presence in Milk of the Extrinsic Factor of Castle, Proc. Soc. Exper. Biol. & Med. 37: 149, 1937.
30. Singer, K.: Eiertherapie der perniziösen Anämie, Wien. klin. Wchnschr. 45: 1063, 1932.
31. Miller, D. K., and Rhoads, C. P.: The Presence in Egg White and in a Rice Polishings Concentrate Low in Vitamin G of an Anti-Pernicious Anemia Principle, New England J. Med. 211: 921, 1934.
32. Reimann, F.: Versuche zur Potenzierung der Wirkung oral verabreichter Leber. Kurzo wissenschaftliche mitteilung, Med. Klin. 27: 880, 1931.
33. Strauss, M. R., and Castle, W. B.: The Nature of the Extrinsic Factor of the Deficiency State in Pernicious Anemia and in Related Macrocytic Anemias, New England J. Med. 207: 55, 1932.
34. Castle, W. B.: The Etiology of Pernicious Anemia and Related Macrocytic Anemias, Ann. Int. Med. 7: 2, 1933.
35. Castle, W. B., and Ham, T. H.: Further Evidence for the Essential Participation of Extrinsic Factor in Hematopoietic Responses to Mixtures of Beef Muscle and Gastric Juice and to Hog Stomach Mucosa, J. A. M. A. 107: 1456, 1936.
36. Castle, W. B., Ross, J. B., Davidson, C. S., Burehenal, J. H., Fox, H. J., and Ham, T. H.: Extrinsic Factor in Pernicious Anemia: Ineffectiveness of Purified Casein and of Identified Components of the Vitamin B Complex, Science 144: 81, 1944.
37. Formijne, P.: Experiments on the Properties of the Extrinsic Factor and on the Reaction of Castle, Arch. Int. Med. 66: 1191, 1940.
38. Castlo, W. B., and Rhoads, C. P.: The Etiology and Treatment of Sprue in Porto Rico, Lancet 1: 1198, 1932.
39. Castle, W. B., Rhoads, C. P., Lawson, H. A., and Payne, G. C.: Etiology and Treatment of Sprue. Observations on Patients in Puerto Rico and Subsequent Experiments in Animals, Arch. Int. Med. 56: 627, 1935.
40. Foy, H., and Kondi, A.: Response of Nutritional Macrocytic Anaemia to Anahaemin, Lancet 2: 360, 1939.
41. Wills, L., and Evans, B. D. F.: Tropical Macrocytic Anemia: Its Relation to Pernicious Anemia, Lancet 2: 416, 1938.
42. Napier, L. E., and Others: Etiology of Tropical Macrocytic Anaemia, Indian M. Gaz. 74: 1, 1939.
43. Fairley, N. H.: Tropical Macrocytic Anaemia in an Indian Treated With Anahaemin, Lancet 1: 118, 1940.
44. Trowell, H. C.: Liver Extract in Treatment of Tropical Macrocytic Anemia, Lancet 2: 303, 1941.
45. (a) Hawk, P. B., and Bergeim, O.: Practical Physiological Chemistry, ed. 11, Philadelphia, 1937, P. Blakiston's Son and Co., Inc., p. 378.
 (b) Saxon, G. J.: A Method for the Determination of the Total Fats of Undried Feces and Other Moist Masses, J. Biol. Chem. 17: 99, 1914.
 (c) Smith, C. A., Miller, R. C., and Hawk, P. B.: Changes in the Fat Content of Feces Preserved by Freezing Without the Addition of a Preservative, J. Biol. Chem. 21: 395, 1915.
46. Mackie, T. T., Miller, D. K., and Rhoads, C. P.: Sprue: Roentgenologic Changes in Small Intestine, Am. J. Trop. Med. 15: 571, 1935.
47. Kantor, J. L.: The Roentgen Diagnosis of Idiopathic Steatorrhea and Allied Conditions, Am. J. Roentgenol. 41: 758, 1939; Arch. Int. Med. 65: 988, 1940.

48. Wintrobe, M. M.: Antinemic Effect of Yeast in Pernicious Anemia, *Am. J. M. Sc.* 197: 286, 1939.
49. Ungley, C. C., and James, G. V.: The Effect of Yeast and Wheat Embryo in Anemias. II. The Nature of the Hematopoietic Factor in Yeast Effective in Pernicious Anemia, *Quart. J. Med.* 3: 323, 1934.
50. Grinker, R. R.: Pernicious Anemia, Achylia Gastrica and Combined Cord Degeneration and Their Relationship, *Arch. Int. Med.* 38: 291, 1926.
51. Castle, W. B., Heath, C. W., and Strauss, M. B.: Observations on the Etiologic Relationship of Achylia Gastrica to Pernicious Anemia IV, *Am. J. M. Sc.* 182: 741, 1931.
52. Wilkinson, J. F.: The Gastric Secretion in Pernicious Anemia, *Quart. J. Med.* 1: 361, 1932.
53. Harvey, E. A., and Murphy, W. P.: Pernicious Anemia Without Achlorhydria, *Ann. Int. Med.* 6: 1393, 1933.
54. Levin, A. L.: The Occurrence of a Pernicious Anemia Syndrome in the Presence of Normal Gastric Acidity: Report of an Instance, *Am. J. Digest Dis.* 1: 240, 1934.
55. Alsted, G.: On Free Hydrochloric Acid in the Stomach in Pernicious Anemia, *Acta med. Scandinav.* 82: 288, 1934.
56. Finney, J. O.: Pernicious Anemia Unassociated With Achlorhydria: Case Report, *Ann. Int. Med.* 12: 1521, 1939.
57. Connery, J. E., and Jolliffe, N.: Studies on the "Acid Deficit" in Pernicious Anemia, With Report of a Case Showing Return of Free Acid, *Am. J. M. Sc.* 181: 830, 1931.
58. Davidson, L. S. P.: Pernicious Anemia With Return of Hydrochloric Acid and Ferments After Treatment, *Brit. M. J.* 1: 182, 1933.
59. Chevallier, P., Gutmann, R. A., Salmon, A. R., and Fiehrer, A.: Sur la reprise très rapide de la sécrétion gastrique dans la maladie de Biermer traitée par le foie, *Sang.* 7: 756, 1933.
60. Miller, D. K., and Rhoads, C. P.: The Experimental Production of Loss of Hematopoietic Elements of the Gastric Secretion and of the Liver in Swine With Achlorhydria and Anemia, *J. Clin. Investigation* 14: 153, 1935.
61. Rodriguez-Molina, R.: Hematology of Sprue, Puerto Rico *J. Pub. Health & Trop. Med.* 15: 89, 1939.
62. Rhoads, C. P., and Castle, W. B.: The Pathology of the Bone Marrow in Sprue Anemia, *Am. J. Path.* 15: 483, 1933.
63. Hanes, F. M.: Diagnostic Criteria and Resistance to Therapy in the Sprue Syndrome, *Am. J. M. Sc.* 204: 436, 1942.
64. Miller, D. K., and Baker, W. H.: Clinical Course and Treatment of Sprue, *Arch. Int. Med.* 60: 385, 1937.

PROPYLENE GLYCOL AS A MENSTRUUM FOR THE ADMINISTRATION OF STEROID HORMONES

THOMAS HODGE MCGAVACK, M.D.,* AND MILDRED VOGEL, M.S.†
NEW YORK, N. Y.

THE low toxicity of propylene glycol has been confirmed by a number of investigators.¹⁻⁷ Hanzlik and his associates² found it impossible to produce death from a single dose administered orally. Rapid injection of the substance in dogs produced a fatal result only when as much as 25 c.c. of a 50 per cent solution in physiologic saline were given per kilogram of body weight. Chronic intoxications have been even more difficult to produce. Morris, Nelson, and Calvery⁵ fed the material to albino rats for two years and noted that the animals "differed only very slightly from the controls." Seidenfeld and Hanzlik¹ fed substantial amounts to rats during approximately one-eighth of their life span without any appreciable effect.

When all of the carbohydrate of the diet was replaced by propylene glycol (representing 48.5 per cent of the calories in the diet), young rats failed to gain weight and died within approximately one month.² When three-fourths of the carbohydrate was similarly replaced, the animals lived three months. And, if the proportions of propylene glycol to carbohydrate were as 1:1 (representing a daily ingestion of approximately 30 c.c. of propylene glycol per animal), no death was noted under five months of feeding. If one-eighth of the dietary carbohydrates was replaced by propylene glycol, a good gain in weight was observed, nearly equal to that of the controls, and no deaths were seen under five months of treatment. If one-eighth of the diet was replaced, the animals reacted as did the controls. If one-eighth of the weight of the normal diet was fed as propylene glycol in addition to the diet, the treated animals gained more rapidly than did the controls, an effect attributable to the caloric value of the added propylene glycol. The available evidence justifies the conclusion that propylene glycol is comparatively nontoxic and that, even in relatively large amounts, it can be utilized by the body as a source of energy.

Pathologic changes observed as occurring in fatal intoxications with propylene glycol have been limited to the liver and kidneys. In the former, moderate destruction of liver cells with some karyolysis has been noted, while the latter have shown degenerative changes in the cells of the convoluted tubules. However, many of the animals sacrificed after five months on a diet in which one-half of the carbohydrate was replaced by propylene glycol have shown no histologic alterations whatsoever.¹

From the Department of Medicine, New York Medical College, and the Research Unit, Metropolitan Hospital.

Received for publication, Aug. 12, 1944.

*Associate Professor of Medicine, New York Medical College.

†Assistant in Research, Research Unit of Metropolitan Hospital.

The relative ease with which propylene glycol can be converted by oxidation into lactic acid probably accounts for its low toxicity and for most of its metabolic effects. The substance increases glycogen storage.⁶ If it be given in large quantities, about two-thirds is metabolized as carbohydrate, and one-third is excreted unchanged.⁷ Presumptively, as a result of its carbohydrate-like behavior, animals treated with the drug show an increased activity and decreased fatigability.^{3, 6} However, the basal metabolic rate has not been increased, either in animals or in human beings.

Because of its low toxicity and the ability of the animal body to utilize quantities of the drug as food, propylene glycol has been proposed as a vehicle for water-insoluble medicaments, particularly if they are to be administered intravenously. In this connection the purpose of the present study is three-fold: (1) to determine the toxic action, if any, of moderate amounts of intramuscularly or intravenously administered propylene glycol; (2) to study the effects of intravenously administered desoxycorticosterone acetate upon the intact subject, using propylene glycol as a vehicle; and (3) to attempt the production of thrombi as a result of the intravenous use of desoxycorticosterone acetate solutions in propylene glycol.

METHODS AND MATERIALS

Seven male hospital patients selected at random and seven dogs acclimated for from three to four weeks prior to the experimental period served as subjects of the study. Intramuscular injections were given in the gluteal region. The antecubital veins were utilized for injections in the human patients, while both neck and leg veins were used in the dogs; in man, care was taken to avoid repetition of the injections into a single vein on successive days.

Except for slight modifications described elsewhere,¹⁹ the methods detailed in the references indicated were used, and estimations were made of the following components of the blood on all subjects before, during, and at the end of, the experimental period: sodium,⁸ potassium,⁹ chloride,¹⁰ icteric index,¹¹ Van den Bergh,¹² cephalin precipitation,¹³ phosphatase,¹⁴ phosphorus,¹⁵ total¹⁶ and free¹⁷ cholesterol, total proteins,¹⁸ albumin, and globulin.

The animals were killed by the intravenous injection of pentobarbital, about 1 c.c. per kilogram of body weight. Desoxycorticosterone acetate was used in the form of a solution containing 10 mg. in 1 c.c. of propylene glycol.* Organs and other tissues were studied grossly and microscopically.

1. THE EFFECTS OF PROPYLENE GLYCOL FOLLOWING REPEATED INTRAMUSCULAR OR INTRAVENOUS ADMINISTRATION

It has already been shown that approximately 25 c.c. of a 50 per cent solution of propylene glycol in physiologic saline per kilogram of body weight must be injected intravenously into the dog at a rate of not less than 0.5 c.c. per minute in order to produce death.² Inasmuch as such amounts are many times that which would be used as *menstruums* for medicinally active substances, the present study was devoted to ascertaining the influence of repeated injections intramuscularly and intravenously.

*We are indebted to Dr. Max Gilbert, of the Schering Corporation, for generous supplies of this material, as well as for especially prepared ampules of plain propylene glycol.

A. IN THE DOG.—One dog was given twenty-one daily intramuscular injections of 1 c.c. of propylene glycol into the gluteal region of the right hind leg; a similar amount of sesame oil was simultaneously administered into the left hind leg. The dog was sacrificed the day after the last injection, and histologic sections were made through the areas of the injections. Grossly, both the area of propylene glycol administration and that of sesame oil injection showed an increased vascularity. The latter site contained unabsorbed fluid in which free oily particles were readily distinguishable. Microscopic examination disclosed in the area of the sesame oil injections large numbers of fat globules between the individual muscle cells and bundles. Sections taken from the site of the propylene glycol injections showed some disruption of the muscle bundles with infiltration of large numbers of monocytes, plasma cells, lymphocytes, and scattered groups of red blood cells and polymorphonuclear leucocytes. No foreign material, such as fat, was present.

At autopsy there was no evidence either grossly or microscopically of pathologic changes in the liver, thymus, kidneys, pancreas, adrenals, thyroid, and gonads, and the weights of all were normal. An analysis of the kidney, spleen, and diaphragm for 17-ketosteroids showed only traces present, and none was found in the lung.

A second dog (Dog C) was given 1 c.c. of propylene glycol intravenously every day for forty-three consecutive injections. No specific effects could be detected. During the period of injections, the dog gained from 6.9 to 7.2 kg. in weight. At the time of sacrifice there was no gross or histologic evidence of pathologic alterations in any organ. Normal values for blood chemical constituents before and after treatment are shown in Table I.

Three dogs weighing 5.9, 6.3, and 8.1 kg., respectively, were each given 20 c.c. of propylene glycol daily for three successive days. Immediately following the last injection, they were sacrificed. No gross or microscopic alterations could be found in any of the organs or other tissues of the body. Urine obtained from the bladder immediately post mortem showed no albumin or sugar and no abnormal constituents in the sediment.

B. IN THE HUMAN BEING.—Each of four patients was given sixteen intramuscular injections of propylene glycol on successive days. These patients were selected at random; one was convalescing from a rather massive pulmonary infarct secondary to cardiac disease; one had cirrhosis of the liver; one had arthritis; and the fourth suffered from pituitary infantilism. During the first five days of injections, the doses were 0.5, 0.5, 1, 2, and 3 c.c., respectively, following which 3 c.c. were given daily. One patient complained of a slight burning sensation over the area of the injection lasting for from one to two hours on one occasion. The others had neither subjective symptoms of discomfort nor objective signs of injury during or following any of the injections.

Following this series of injections, the same four patients were given propylene glycol intravenously for eleven consecutive days. Dosage was begun with 1 c.c. and increased at the rate of 1 c.c. daily until 10 c.c. had been given. For these injections, syringes were boiled in the usual manner. One patient complained of a sense of warmth in the axilla if the injection were given rapidly. In no other instance was there any demonstrable local or systemic reaction, irrespective of variations in the amount or speed of the infusion.

No abnormal alterations were observed in the icteric index, Van den Bergh reaction, or cephalin flocculation in any patient. There was no disturbance in the blood sodium, chloride, potassium, phosphorus, total or free cholesterol, phosphatase, or protein partition as a result of either the intramuscular or intravenous injection of the propylene glycol.

CONCLUSION.—Propylene glycol is a relatively nontoxic material which can be given repeatedly to dogs or human beings in relatively large doses, either intramuscularly or intravenously, without any signs of untoward reaction except slight local injury when given intramuscularly. It should prove to be a satisfactory menstruum for medicinal agents which are relatively insoluble in water or watery solutions.

II. THE EFFECTS IN INTACT ANIMALS AND IN HOSPITAL PATIENTS OF INTRAVENOUSLY ADMINISTERED DESOXYCORTICOSTERONE ACETATE IN PROPYLENE GLYCOL

Desoxycorticosterone acetate dissolved in propylene glycol has been administered to dogs subcutaneously,²⁰ intramuscularly,²⁰ and intravenously,²¹ but not to human beings by any of these three routes. The present studies were designed primarily to determine the degree of risk, if any, involved in the use of this preparation intravenously.

A. EXPERIMENTS ON DOGS.—Six animals were given desoxycorticosterone acetate in propylene glycol intravenously in varying doses over widely variable periods of time. All animals gained weight during the treatment and remained in good health. Diarrhea appeared for a short period of time in two animals (Dogs 4 and 6), but this could not be related with certainty to the action of the drug, as it disappeared in both instances without any known alteration in the experimental regime.

1. *The Effect of a Single Intravenous Injection.*—Dog 2 was given a single intravenous injection of 50 mg. of desoxycorticosterone acetate (5 c.c. of propylene glycol containing 50 mg. of desoxycorticosterone acetate) and was sacrificed four and one-half hours later. There was no change in behavior during or after the injection. Laboratory data are given in Table I. The significant features noted were an increase in the sodium and chloride contents of the blood with a simultaneous decrease in the concentration of potassium in the blood. When the dog was sacrificed four and one-half hours after the injection of desoxycorticosterone acetate, there was no gross or histologic evidence of organic damage, nor did the kidneys, spleen, diaphragm, or lung contain more than "traces" of 17-ketosteroids. Particular attention was paid to the presence or absence of thrombi, but none was found even after careful microscopic search.

2. *The Effects of Repeated Intravenous Injections.*—Five dogs were given daily intravenous injections of desoxycorticosterone acetate in propylene glycol, in doses varying from 10 to 200 mg. over periods varying from six to forty-seven days. The laboratory data from these experiments is summarized in Table I.

a. *Behavior in Relation to the Individual Injection:* Injections were given as rapidly as the propylene glycol solution of desoxycorticosterone acetate would pass through a 22 gauge needle. No effect of any kind could be elicited in any animal receiving less than 150 mg. at a single dose. In Dog 4, the

TABLE I

DATA FROM DOGS RECEIVING INTRAVENOUS INJECTIONS OF DESOXYCORTICOSTERONE ACETATE IN PROPYLENE GLYCOL

DOO	DAY OF EXPERIMENT	WEIGHT (KG.)	D C A* (NO.)		ICTERIC INDEX	VAN DEN BERGH	PHOSPHATASE†	CEPHALIN PRECIPITATION	BLOOD CHEMICAL VALUES (NO. PER 100 C.C.)						A/O RATIO
			DAILY	TOTAL TO DATE					K	Cl‡	CHOLESTEROL		TOTAL		
											Na	FREE			
C	0	6.9	0	0	3.9	Neg.	9.7	++++	---	---	100	28	5.40	1.82	
	43	7.2	0	0	5.0	Neg.	5.8	++++	660	660	187	52	7.10	1.90	
1	0	11.6	0	0	5.0	Neg.	3.0	++++	---	---	100	28	5.16	1.77	
	38	13.0	10	270	5.1	Neg.	3.4	++++	13.0	550	200	52	7.80	3.00	
	47	13.8	10	330	5.7	Neg.	3.9	++++	12.6	586	190	44	7.20	1.40	
2	0	8.7	0	0	5.0	Neg.	5.7	+++	14.3	624	170	48	7.02	2.18	
	4.5	---	50	50	5.0	Neg.	3.8	++++	6.9	668	180	60	7.00	2.19	
3	0	6.6	0	0	4.0	Neg.	5.0	++++	---	---	110	30	5.40	1.57	
	25	6.9	10	80	---	---	---	----	---	---	---	---	---	---	
43	0	7.0	20	300	5.0	Neg.	5.0	++++	4.9	799	180	42	5.88	2.13	
	0	6.9	0	0	5.5	Neg.	3.0	++++	5.6	636	176	70	8.00	1.66	
5	17	7.5	200	2,890	5.0	Neg.	4.3	++++	5.8	685	130	46	8.50	0.85	
	0	9.5	0	0	3.3	Neg.	5.5	++++	12.4	602	170	50	6.12	2.06	
7	0	9.9	50	300	5.0	Neg.	5.3	++++	8.3	685	210	40	6.48	2.00	
	0	7.8	0	0	5.5	Neg.	7.0	+++	10.6	595	138	40	6.60	1.74	
14	0	8.2	100	1,300	5.0	Neg.	4.3	++++	12.0	602	145	42	7.50	1.83	

*Desoxycorticosterone acetate, 10 mg. per 1 c.c. of propylene glycol was used in all experiments. To the control dog (Dog C) 1 c.c. of plain propylene glycol was given intravenously daily for forty-three days. Theoretically: total dose = daily dose X number of days of the experiment. The discrepancies noted represent days when no injection was given; for example, Sundays.

†Bodansky units.

‡As sodium chloride.

§The sum of the albumin and globulin fractions has been accepted as the total figure.

||Represents hour of experiment.

injection usually took approximately two minutes. By the time 15 to 17 c.c. of the solution had entered the circulation, the dog routinely developed convulsive seizures which lasted for from one-half minute to three minutes. Following this, he would usually vomit. A convulsion could be avoided entirely by changing the time taken to inject the drug from two to three and one-half minutes. Although vomiting still occurred occasionally, it seemed to be unrelated to the speed of the injection. These reactions did not prevent a gain in weight, nor did they seem to interfere with hepatic function (Table I).

b. *Alterations in Blood Chemical Findings:* In none of the animals were there any changes in the following as a result of the treatment described: icteric index, Van den Bergh reaction, cephalin precipitation, phosphatase, phosphorus, total cholesterol, free cholesterol, albumin, or globulin (Table I). The sodium and chloride concentrations in the blood behaved in a somewhat unexpected manner. There was a significant elevation: four and one-half hours after a single injection of 50 mg. (Dog 2, Table I), five hours after a single injection of 200 mg. (Dog 4, Table I), and after six daily injections of 50 mg. each (Dog 3, Table I; Dog 5, Table I). In Dog 4, which showed a marked rise after a single injection, sodium and chloride values were still elevated at the end of fifteen daily injections, after a total of 2,890 mg. of drug had been administered, but the level was distinctly lower than it had been following the first dose. In Dogs 1 and 6, the values after treatment were normal and, in the latter animal, corresponded closely to those obtained in the "fore" period. Toward the end of the experimental period, both of these animals drank more water, but circumstances did not permit a careful measurement of intake or output. Potassium values were usually lower at the end of the experiments than at the beginning, but the magnitude of the changes was not significant.

c. *Local Reaction to the Injections:* In and around the site of repeated injections of propylene glycol or desoxyeorticosterone in propylene glycol there was always a fibrous tissue reaction with thrombus formation, often ending in the complete obliteration of the lumen of the vessel. Unless the surrounding tissues had been infiltrated during injection, there was no histologic evidence of any deposit of desoxyeorticosterone acetate in these areas.

d. *Pathologic Study of All Animals:* No gross abnormalities could be detected in any of the organs or tissues of either the control or experimental animals with the exception of the site of the repeated intravenous injections, which showed the perivascular induration and thrombus formation mentioned in the preceding paragraph. Weights of the liver, kidneys, gonads, thyroid, pancreas, and adrenals were normal in both control and experimental dogs when analyzed on the basis of organ weight-body weight ratios. This statement justifies particular emphasis in relation to the adrenal gland.

Microscopic examination showed no difference between control and test subjects except in the case of Dog 5, which received approximately 200 mg. of desoxyeorticosterone acetate daily for fifteen days and which on several occasions developed convulsions during the injection. In the lung of this animal there were scattered clumps of a deep brownish material, which was perivascular in one section, but had no obvious relationship to any tissue ele-

ment in numerous other sections. This material was believed to be desoxycorticosterone acetate, as the same histologic reaction was observed following its purposeful injection into periarterial tissue, in which it was later identified by chemical determination. However, there were no intravascular deposits of this material, nor were there any thrombi present in any part of the lung or in any other organ or tissue of the body.

e. *The Chemical Determination of the 17-Ketosteroids in Body Tissues:* The total 17 ketosteroids were estimated in the following tissues of four dogs (Dogs 2, 4, 5, and 6) receiving desoxycorticosterone acetate: kidney, spleen, diaphragm, lung, liver, and heart. Traces could be detected in all the tissues except the lung of Dog 2 which received a single injection of 50 mg. of the drug four and one-half hours before it was sacrificed. The heart of this animal was not examined. In the other three animals, traces only were present in all the tissues examined except the heart. Here the amount could be determined quantitatively and was as high as 0.7 mg. per 100 Gm. of tissue in Dog 6. Histologic studies of the hearts were not made.

B. STUDIES IN HUMAN BEINGS.—Three patients with cirrhosis of the liver were used for these studies. At the time of the injections, two were severely ill with ascites and ankle edema, while the third was well compensated and nearly ready for discharge from the hospital. The first two showed abnormal liver function as determined by the following studies upon the blood: ieteric indices, Van den Bergh reactions, cephalin flocculation, phosphatase, cholesterol partition, and protein distribution. The third showed normal values for all these. Each received twelve injections on successive days, the first two representing 0.5 and 1 c.c., respectively, of the standard solution, and the remaining ten, 1.5 c.c. each. There was no increase in the ascites or edema of the two decompensated cirrhotics, no increase in blood pressure, and no untoward immediate or late reaction to the injections in any instance. In the third individual, there was no increase in weight, no tendency for the development of edema in the abdomen or extremities, and no change in blood pressure. Values for the constituents of the blood mentioned above showed no alteration as a result of the injections. Sodium, potassium, and chloride studies were not done.

DISCUSSION AND CONCLUSIONS.—The above findings would suggest that relatively large intravenous doses of desoxycorticosterone acetate can be compensated for in the intact animal, provided salt is not forced; for, after a transient period of elevation in blood sodium and chloride levels, these became and remained normal despite rather massive doses of the drug. Compensation for such large amounts of the steroid does not appear to be associated with histologic alterations or gross change in the weight of the adrenal glands; nor it is accompanied by an enlargement or dilatation of the heart. That the effects are not the result of inactivation of the hormone as a result of its intravenous injection has been adequately shown by Cleghorn,²¹ who maintained two adrenalectomized dogs "indefinitely" through the intravenous administration of desoxycorticosterone acetate in propylene glycol. The application of the drug in patients was not sufficiently prolonged to bring out critically adverse effects, but suggests that, even where the liver is damaged, the body

with normal adrenals is capable of compensatory responses which, at least for a short time, neutralize or prevent the full exhibition of the powerful action of the drug.

The absence of desoxycorticosterone acetate from the area of thrombus formation about the site of the intravenous injection suggests that the effect obtained was a nonspecific one which could have been produced as readily by other hypertonic solutions.

When injected intravenously, no untoward clinical reactions were caused by single injections of desoxycorticosterone acetate in doses of 100 mg., representing 10 c.c. of propylene glycol, whereas nausea and vomiting appeared if 200 mg. were similarly given. Moreover, generalized convulsions could be produced by increasing the speed of the injection. These amounts of drug are from five to ten times as great as those therapeutically indicated in Addison's disease. It seems fair to conclude that the drug is safe for intravenous application in the crisis of that disease.

III. THE PROBLEM OF EMBOLIZATION FOLLOWING THE INTRAVENOUS USE OF SOLUTIONS OF DESOXYCORTICOSTERONE ACETATE IN PROPYLENE GLYCOL

Propylene glycol is a very satisfactory solvent for desoxycorticosterone acetate, dissolving 10 mg. or more per cubic centimeter. When such solutions are diluted with water, precipitation of the drug occurs immediately. It has, therefore, been postulated that the intravenous use of propylene glycol solutions of desoxycorticosterone acetate would be fraught with the danger of embolization as a result of a rather rapid precipitation of the chemical. Such a conclusion has not been borne out by work with dogs, provided, as noted in section I, the amounts of drug introduced remained below 200 mg., represent-

TABLE II

THE EFFECT OF SALINE DILUTION UPON THE PRECIPITATION AND CRYSTALLIZATION OF DESOXYCORTICOSTERONE ACETATE FROM SOLUTION IN PROPYLENE GLYCOL

NUMBER OF TRIALS	AMOUNT OF DCA* (MG.)	AMOUNT OF SALINE (C.C.)	DILUTION	PRECIPITATE†			
				AMORPHOUS	AVERAGE TIME TO APPEAR (SEC.)	CRYSTAL-LINE	AVERAGE TIME TO APPEAR (SEC.)
3	0.5	0.5	1: 1,000	++++	Immed.	++++	23
4	0.5	1.0	1: 2,000	++++	Immed.	++++	28
4	0.5	2.0	1: 4,000	++++	Immed.	++++	52
8	0.5	2.5	1: 5,000	++++	Immed.	++++	46
4	0.5	4.0	1: 8,000	+++	Immed.	++++	99
5	0.5	5.0	1:10,000	+++	Immed.	++++	78
4	0.5	6.0	1:12,000	+++	Immed.	+++	51
4	0.5	7.0	1:14,000	+++	Immed.	+++	82
4	0.5	8.0	1:16,000	++	Immed.	++	122
4	0.5	9.0	1:18,000	++	Immed.	++	144
4	0.5	10.0	1:20,000	+	Immed.	+	150
4	0.5	12.0	1:24,000	±	Immed.	±	135
3	0.5	14.0	1:28,000	±	Immed.	±	240
4	0.5	16.0	1:32,000	±	10	±	360
4	0.5	18.0	1:36,000	±	10	±	360
4	0.5	20.0	1:40,000	?	10	0	24 hr.

*Represents a 1 per cent solution of desoxycorticosterone acetate in propylene glycol.

†The plus signs represent a quantitative grading of both the "amorphous" and "crystal-line" precipitates. In the case of the latter they also have qualitative significance as follows: +, small crystals; ++, very large crystals and "bundles" or chains of crystals; +++ and +, small crystals, "sheaf" and "chain" formations retained; ±, small crystals not seen in "bundles" or "chains"; 0, no crystals found.

ing 20 c.c. of solution. Moreover, in such doses a rapid injection was necessary for the production of signs and possibly important for the deposition of the material in the lungs.

For these reasons, some phenomena associated with the mixing of propylene glycol solutions of desoxycorticosterone acetate with watery solutions have been observed *in vitro*. These are summarized in Table II. The reactions were similar whether distilled water or physiologic saline was employed.

When the propylene glycol-desoxycorticosterone acetate solution and water were mixed in dilutions up to 1:28,000, a flocculate appeared immediately, which, under the microscope, was shown to consist of amorphous particulate matter of rather uniform size, approximately 3 to 5 microns in diameter. In a dilution of 1:40,000 or above, this material has failed to show crystallization over periods up to twenty-four hours, whereas with greater concentrations, needlelike crystals varying in length from 9 to 60 microns rapidly made their appearance, being present in about thirty seconds in dilutions of 1:1,000. In dilutions up to 1:20,000, all of the precipitated material changes to a crystalline form within a very few minutes. This process was hastened by agitation, and eventually large floccula up to 1 or 2 cm. in length could be obtained in the higher concentrations.

DISCUSSION AND CONCLUSIONS.—It is obvious from the above experiments that desoxycorticosterone acetate precipitates from propylene glycol solutions in an amorphous form at first and remains in that form if sufficiently diluted. The individual particles of this precipitate conform quite satisfactorily to the size and shape of other particulate matter in the blood stream. However, if they are allowed to stand without too much dilution, acicular crystals result, which conceivably might act as emboli.

The bedside application of these facts is important. In all our injections of propylene glycol solutions of desoxycorticosterone acetate into patients, we have used "wet-sterilized" syringes. Microscopic examination of the interfaces of solution and droplets of water in the syringe has routinely shown crystals of the material. Despite this, no palpable reaction from the injections has been observed. In six dogs receiving similarly prepared injections, no evidence of emboli could be detected grossly or microscopically. In one of these, with very large doses, precipitation of the material did occur within the lung, but without embolus or thrombus formation. In view of the above, we believe the intravenous injection of solutions of desoxycorticosterone acetate in propylene glycol (10 mg. per 1 c.c.) is a safe procedure within any known therapeutic range of dosage. However, in applying the drug in this fashion, it is important to observe the following rules:

1. An empty syringe should be used for inserting the intravenous needle. As soon as the needle is shown to be in the vein, a syringe containing the desoxycorticosterone acetate solution should be attached and positive pressure immediately established and maintained throughout the injection.
2. Syringes for injecting the solution should be "dry-sterilized" to avoid any precipitation of the active ingredient.
3. The material should be injected slowly, not to exceed a rate of 2.5 c.c. per minute. This assures its rapid dilution and should prevent the formation of any acicular precipitate.

REFERENCES

1. Seidenfeld, M. A., and Hanzlik, P. J.: The General Properties, Actions, and Toxicity of Propylene Glycol, *J. Pharmacol. & Exper. Therap.* 44: 109, 1932.
2. Hanzlik, P. J., Newman, H. W., Van Winkle, W., Jr., Lehman, A. J., and Kennedy, N. K.: Toxicity, Fate, and Excretion of Propylene Glycol and Some Other Glycols, *J. Pharmacol. & Exper. Therap.* 67: 101, 1939.
3. Hanzlik, P. J., Lehman, A. J., Van Winkle, W., Jr., and Kennedy, N. K.: General Metabolic and Glycogenic Actions of Propylene Glycol and Some Other Glycols, *J. Pharmacol. & Exper. Therap.* 67: 114, 1939.
4. Lehman, A. J., and Newman, W. H.: Propylene Glycol: Rate of Metabolism, Absorption, and Excretion, With a Method for Estimation in Body Fluids, *J. Pharmacol. & Exper. Therap.* 60: 312, 1937.
5. Morris, H. J., Nelson, A. A., and Calvery, H. O.: Observations on the Chronic Toxicities of Propylene Glycol, Ethylene Glycol, Diethylene Glycol, Ethylene Glycol Mono-Ethyl-Ether, and Diethylene Glycol Mono-Ethyl-Ether, *J. Pharmacol. & Exper. Therap.* 74: 266, 1942.
6. Van Winkle, W., Jr., and Kennedy, N. K.: Voluntary Running Activity of Rats Fed Propylene Glycol and Other Glycols, *J. Pharmacol. & Exper. Therap.* 69: 140, 1940.
7. Van Winkle, W., Jr.: Quantitative Gastrointestinal Absorption and Renal Excretion of Propylene Glycol, *J. Pharmacol. & Exper. Therap.* 72: 344, 1941.
8. Darnell, M. C., Jr., and Walker, B. S.: Determination of Sodium in Biological Fluids, *Indust. & Engin. Chem. Analyt. Ed.* 12: 242, 1940.
9. Dreker, I. J.: In press.
10. Wilson, D. W., and Ball, E. G.: A Study of the Estimation of Chloride in Blood and Serum, *J. Biol. Chem.* 79: 221, 1928.
11. Meulengracht, E.: Blood Sugar Curves in Various Forms of Icterus, *Acta med. Scandinav.* 79: 32, 1932.
12. Peters, J. P., and Van Slyke, D. D.: Quantitative Clinical Chemistry, Baltimore, 1932, Williams & Wilkins, vol. 2.
13. Hanger, F. M.: Flocculation of Cephalin-Cholesterol Emulsions by Pathological Sera, *Tr. A. Am. Physicians* 53: 148, 1938.
14. Bodansky, A.: Phosphatase Studies. II. Determination of Serum Phosphatase. Factors Influencing the Accuracy of the Determination, *J. Biol. Chem.* 101: 93, 1933.
15. Fiske, C. H., and Subbarow, Y.: The Colorimetric Determination of Phosphorus, *J. Biol. Chem.* 66: 375, 1925.
16. Schoenheimer, R., and Sperry, W. M.: A Micromethod for the Determination of Free and Combined Cholesterol, *J. Biol. Chem.* 106: 745, 1934.
17. Dreker, I. J.: A New Method for the Direct Determination of Cholesterol, *Bull. New York M. Coll., Flower & Fifth Ave. Hosps.* 6: 133, 1943.
18. Todd, J. C., and Sanford, A. H.: Clinical Diagnosis by Laboratory Methods, ed. 10, Philadelphia, 1943, W. B. Saunders Co., p. 377.
19. Schwimmer, D., Klotz, S., Dreker, I. J., and McGavneek, T. H.: *Ann. J. Digest. Dis.* (In press.)
20. Winkler, A. W., Smith, P. K., and Haff, H. E.: Absence of Beneficial Effects From Injections of Desoxycorticosterone Acetate and of Cortical Adrenal Extract in Experimental Anuria, *J. Clin. Investigation* 21: 419, 1942.
21. Cleghorn, R. A., Clarke, A. P. W., and Greenwood, W. F.: Activity of Desoxycorticosterone Acetate in Propylene Glycol by Oral and Intravenous Routes in Adrenalectomized Dogs, and Its Effect on the Cardiac Arrhythmia of Adrenal Insufficiency, *Endocrinology* 32: 170, 1943.

CLINICAL CHEMISTRY

A PRACTICAL METHOD FOR THE DETERMINATION OF BLOOD VOLUME WITH THE DYE T-1824*

A SURVEY OF THE PRESENT BASIS OF THE DYE-METHOD AND ITS CLINICAL APPLICATIONS

MAGNUS I. GREGERSEN

THE purpose of the present report is to make available for general use a practical method of measuring blood volume that was developed primarily for the Armed Forces.

During the past sixty to seventy years numerous methods have been devised for measuring blood volume on intact animals or man (for example, dilution of the blood after large intravenous infusions¹; replacement methods¹; methods for measuring plasma volume from the dilution of slowly diffusible foreign substances such as foreign proteins,²⁻⁴ vital dyes,⁵ gum acacia,⁶ and hemoglobin^{7, 8}; the carbon monoxide^{9, 10} and radioactive iron,¹¹ and the radioactive phosphorus techniques for measuring the total erythrocyte volume^{12, 13}). Of these, the dye methods have held the greatest promise of being suitable for routine clinical use, and the original vital red method brought forward by Keith, Rowntree, and Geraghty⁵ was actually simple enough to meet this need. While observations of great value were made with the Keith-Rowntree technique in its original form, experimental studies gradually disclosed that it was far from foolproof. Efforts to eradicate the sources of error and to satisfy certain theoretical objections to the method have led to numerous modifications (for examples, use of other dyes; attempts to improve the accuracy of the colorimetric determinations by employing various types of compensating colorimeters, spectrophotometers or photoelectric colorimeters instead of the simple colorimeter; corrections for hemolysis in the samples; extraction of the dye from the plasma samples; calculation of plasma volume from extrapolation of a one- to two-hour time-concentration curve). As a result the dye method has become a rather elaborate, time-consuming procedure requiring highly specialized and expensive equipment not generally available in clinics. The complicated nature of the technique is not, however, the only consideration which has caused some hesitancy in applying the dye method. Controversies over such fundamental questions as the exact time required for mixing, the criteria of uniform mixing, and

*This work was done partly under grant from the Josiah Macy, Jr., Foundation and partly under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Columbia University.

From the Department of Physiology of the College of Physicians and Surgeons, Columbia University.

Received for publication, Sept. 11, 1944.

whether or not a significant fraction of the dye is lost during the mixing period have created the impression that the method rests upon rather insecure tenets. Furthermore, a few investigators have reported that consistent results could not be obtained with the dye method in shocklike states,^{14, 15} the very conditions in which a reliable determination of blood volume obviously would be of most immediate practical value.

The method which is recommended here is the outcome of several years' work on various aspects of the dye method¹⁶⁻²³ and of more than two years' experience with blood volume measurements in various types of experimental shock in animals¹⁸ and in the study of shock in man.^{24, 25} The assumptions upon which the determinations of blood volume are based have been extensively tested under abnormal as well as normal circulatory conditions and confirmed by factual data. From this evidence it is possible to define the limits of the error introduced by simplifying the determination on man as described below and to show that this error is negligible in comparison with the reduction in blood volume observed in shock caused by trauma or hemorrhage. In view of the conflicts and controversies that have shadowed the dye methods, it has seemed desirable to supplement the description of the equipment and the method with a discussion of those questions which, in the experience of the writer, recur most frequently and which have persisted in placing the dye method in ill repute and in casting doubt upon blood volume measurements in general.

The simplified method described here has special features which makes it possible to determine the blood volume rapidly and with sufficient accuracy for practical purposes.

1. The plasma concentration of the dye T-1824 is measured with the portable Decade Photometer designed by Nickerson.²⁶

2. The total plasma volume is obtained from the dye concentration in a single blood sample drawn ten minutes after the dye injection.

3. The critical and troublesome procedure of measuring out an exact amount of dye at the time of making the determination is eliminated by using ampules containing a standard amount of dye solution of known concentration. This also eliminates all calculations from the determination of total plasma volume. By reference to a chart, the plasma volume is obtained directly from the Decade Photometer reading. The total blood volume is then calculated from the plasma volume and the hematocrit.

4. The hematocrit may be determined with the copper sulfate-specific gravity method²⁷ instead of with a high speed centrifuge. With the addition of the latter technique, all the equipment is readily portable.

A. MATERIALS AND EQUIPMENT

1. *Dye solution (T-1824).*—The dye is supplied in ampules containing exactly 5 c.c. of a solution of T-1824 which has been carefully standardized.* The concentration (approximately 0.45 per cent in water) has been so adjusted that when the solution is diluted 1:500 in human plasma or serum and read at

*These ampules may be obtained from the Warner Institute of Therapeutic Research, New York, N. Y. The preparation on a large scale of ampules containing a fixed amount of dye was made possible through the cooperation of Dr. Marvin Thompson, Director of Research of the Warner Institute.

624 m μ in 10 mm. cells against an identical dye-free blank, its optical density is 0.8.* Each dye ampule is wrapped in a package containing also a 10 c.c. ampule of sterile saline which is needed in transferring the dye solution quantitatively to a syringe (see Procedure).

2. *Syringes*.—One to two dozen 10 c.c. syringes. These are packed and autoclaved in sterilizing tubes (see Fig. 1).

3. *Needles*.—One-half dozen each of 19-, 20-, 21-, and 22-gauge needles for venipuncture. Platinum needles are recommended but not essential. For sharpening the needles use a small Arkansas stone. A pointed needle is preferable to one with a cutting edge. The needles are packed and autoclaved in needle sterilizing tubes (Fig. 1). For arterial punctures, the equipment should also include a 19- or 20-gauge needle of the modified Ungar type,²⁴ in which the inner section can be replaced with a tightly fitting, blunt obturator (Fig. 1).

4. *Stopcocks*.—One or two water-tight, three-way metal stopcocks.

5. *Blood Tubes*.—Pyrex serum tubes of 5 c.c. capacity (80 mm. by 10 mm.) as shown in Fig. 1. In the event that the hematocrit is to be determined by centrifugation rather than with the copper sulfate-specific gravity method, the equipment should also include some form of hematocrit tube, such as the Wintrobe (Fig. 1), which requires only a small amount of blood for the determination. An alternative procedure is to use 4 c.c. hematocrit tubes (100 mm. by 8 mm.)† and utilize the plasma from this source for both dye and plasma protein determinations.

6. *Corks*.—Impregnation with hot paraffin prevents blood from sticking to the corks and minimizes the danger of hemolysis.

7. *Anticoagulant*.—The heparin produced by the Connaught Laboratories‡ is recommended for this purpose. Other dry anticoagulants may be used provided they do not (a) alter the red cell volume, (b) influence the spectral absorption of the dye in plasma, or (c) change the density of the plasma itself.

8. *Pipettes for Collecting Plasma*.—Made from 8 mm. glass tubing drawn out to a fine tip (Fig. 1) and equipped with a small rubber bulb (standard eye dropper bulb).

9. *Screw Cap Jars*.—Three screw cap jars (2 $\frac{7}{8}$ inches in diameter, 3 $\frac{5}{8}$ inches in height). One jar for 70 per cent alcohol, one for sterile gauze, and one for used needles, which is filled with water and contains a rack (plastic) that serves to protect the needle points (Fig. 1).

10. *Charts*.—Used for recording of data (Fig. 2).

11. *Carrying Case*.—9 by 9 by 16 inches (Fig. 3) fitted to hold the items.§

12. *Decade Photometer*.||—Nickerson, 1944²⁵ (Fig. 4).

13. *Portable Outfit (Including Hand Centrifuge)*||.—Used for measuring specific gravity of whole blood and plasma with the copper sulfate method.²⁷

B. DETERMINATION OF BLOOD VOLUME IN NORMAL SUBJECTS

1. The condition of the subject (position, activity, and digestion) influences the plasma volume. Hence it is customary to measure the normal blood volume

*See Appendix for directions for standardizing.

†MacAlaster Bicknell Co., Cambridge, Mass.

‡Connaught Laboratories, University of Toronto, Toronto, Can.

§Fibre Products Manufacturing Co., New York, N. Y.

||Bausch and Lomb Optical Co., Rochester, N. Y.

¶Eimer and Amend, New York, N. Y.

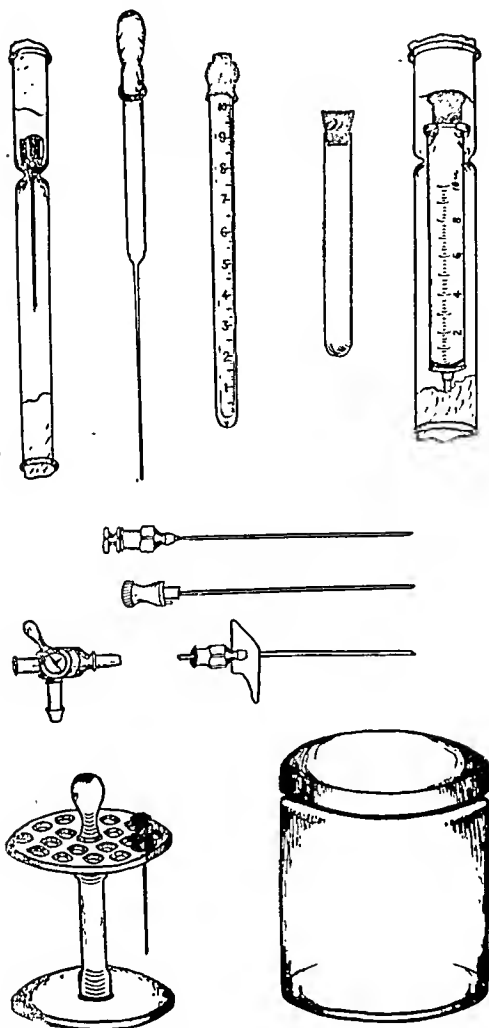


Fig. 1.—Simplified sketch of the principal items included in the blood volume kit showing the construction of the arterial needle. See text for a complete list of materials and equipment.

Blood Volume Determination

Place _____
 Date _____
 Name _____ Serial No. _____ Age _____ Sex M ____ F ____
 Weight _____ Kg. _____ lb.
 Height _____ cm. _____ in. Body Surface _____ sq.M.
 History (e.g., type, extent and time of injury, treatment received, etc.) _____

T-1824 solution: Lot No. _____; D₁ (1:500) _____; c.c. injected _____; vein _____

Procedure: (e.g., technical difficulties, if any, encountered in carrying out the determination, method of rinsing dye syringe, and special circumstances such as lipemia or hemolysis that may influence the result.) _____

TIME	SAMPLE NO.	VEIN ART.	C.C.	DECADE PHOTOMETER READING	HEMATOCRIT ANTICOAGULANT		SPECIFIC GRAVITY		
					TOTAL CELLS	% CELLS	BLOOD PLASMA	% P.P.	% CELLS

Results:

Plasma Volume _____ c.c.; _____ c.c./Kg.; _____ c.c./sq.M.
 Blood Volume _____ c.c.; _____ c.c./Kg.; _____ c.c./sq.M.
 Red Cell Volume _____ c.c.; _____ c.c./Kg.; _____ c.c./sq.M.

Fig. 2.—Protocol sheet indicating the pertinent data that should be recorded with each determination of blood volume.

under reasonably basal conditions. The subject should be in a postabsorptive state* and should remain quiet, lying down for at least fifteen to twenty minutes before and throughout the determination.

2. Attach a 20- or 21-gauge needle snugly to any well-fitted 10 c.c. syringe. Draw up 1 to 2 c.c. of sterile saline, wet the barrel, and expel all air bubbles, leaving the needle as well as the tip of the syringe filled with saline. Being careful to avoid the introduction of air, draw in *all* of the 5 c.c. of dye in the ampule. Follow this with another 1 to 2 c.c. of saline in order to wash the dye in the needle back into the syringe. Detach the needle and expel any air bubbles that may be present.

Do not attempt to rinse the ampule with saline. The amount of dye solution remaining in the ampule has been determined and allowance has been made for

*The lipemia resulting from a recent fatty meal may be troublesome in the dye determination and lead to error, especially if the degree of lipemia is not identical in the dye-free and dye-tinged blood samples. See discussion of this question on page 1275.

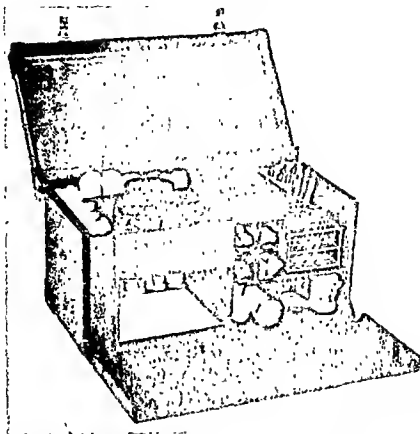


Fig. 3.—Blood volume kit. The lower compartments provide space for several dye units, additional syringes, etc.

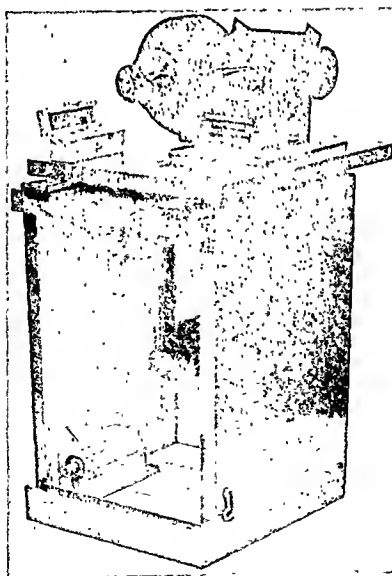


Fig. 4.—Nickerson Decade Photometer (Bausch and Lomb Optical Company, Rochester, New York). The instrument is permanently attached to the door of the carrying case. When open, the case forms a convenient base of such height that when placed on an ordinary table the eyepiece of the instrument is at eye level.

this in the filling of the ampule. Tests have shown that the overall error in this method of giving the dye is within 0.5 per cent.

3. *Without stasis* collect 4 c.c. of blood from the antecubital vein (dye-free sample). Detach the syringe from the needle (leaving the needle in the vein) and empty the blood into a 4 c.c. hematocrit tube or 5 c.c. serum tube (see under 6) containing 1 mg. dry heparin. Cork the tube to prevent evaporation.

Precautions: The practice of making a venipuncture with a single quick jab is not recommended. Go through the skin with a quick thrust, but be deliberate in entering the vein, for if by chance the needle penetrates the opposite wall of the vein, some of the dye may escape during the injection which follows immediately. After the needle is in the vein, wait for one-half to one minute before drawing the sample. By this time the effects of momentary stasis will usually have disappeared.

4. Through the needle already in the vein inject the 5 c.c. of standard T-1824 solution and note the time. Make sure that none of the dye escapes outside the vein. Rinse the syringe several times (three to five) with blood before withdrawing the needle.

5. Exactly ten minutes after the dye injection, collect another 4 c.c. sample of blood *without stasis* from the opposite antecubital vein. Transfer this sample to a second 4 c.c. hematocrit tube or 5 c.c. serum tube containing heparin.

6. Determine the specific gravity of the whole blood with the copper sulfate method.²⁷ After centrifugation of the samples, determine the specific gravity of the plasma. The relative erythrocyte volume as obtained by the conventional hematocrit method is calculated from the equation

$$\text{Per cent erythrocytes} = \frac{(\text{blood sp. gr.}) - (\text{plasma sp. gr.})}{1.0971 - (\text{plasma sp. gr.})}$$

in which 1.0971* is the specific gravity of human red cells. Look for evidence of hemolysis in the supernatant plasma in both tubes (see under D). The hematocrits and plasma protein values may be obtained also by reference to the charts provided by Phillips and co-workers.²⁷

Alternative procedure: If a high speed centrifuge is readily available, the hematocrit may be determined by centrifugation for thirty minutes at 3,000 r.p.m. (radius 15 cm.). Provided the samples are properly taken without stasis, the hematocrits in the dye-free control and dye-tinged samples should agree within 1 or 2 per cent.

7. Collect about 1 c.c. of clear plasma from each blood sample with two narrow-tipped pipettes of the type described. Transfer each plasma sample directly to the appropriate cup of the Decade Photometer. With the dial of the Decade Photometer toward your left, turn the outer dial (coarse adjustment) clockwise until the right-hand field is slightly lighter in color than the left (dye-tinged plasma). Make the fine adjustment by turning the central knob until the two fields match.† Record the number on the dial. This number represents the plasma dye concentration expressed in terms of optical density

*The value 1.1036 will give the true relative cell volume.

†The filters in the coarse adjustment differ in optical density by 0.1 and range from 0.3 to 1.7. The fine adjustment ranges from 0.00 to 0.09 in steps of 0.01.

at 624 $m\mu$. From this value the total plasma volume is obtained directly by referring to the chart* shown in Fig. 5. The total blood volume is then calculated from the following equation:

$$\text{Total blood volume c.c.} = \frac{\text{Plasma volume c.c.}}{1 - \text{hematocrit}}$$

C. DETERMINATION OF BLOOD VOLUME IN PATIENTS IN SHOCK

In shocklike states the peripheral venous circulation is usually poor. The veins are collapsed and blood can be obtained from them only with great difficulty, if at all. In severe cases the veins may be so constricted that fluid can be forced through them only under considerable pressure. Venous blood samples obtained under these conditions are not representative of the circulating blood. In all such instances the samples should be drawn from an artery (brachial or femoral).

Only one arterial puncture need be made. This can be done without pain or discomfort to the patient by infiltration with a little 2 per cent novocain at the site of the puncture. A 19- or 20-gauge needle equipped with a tightly fitting obturator (Fig. 1) is inserted into the brachial artery at the start and left in place. The dye-free and dye-tinged samples can then be drawn without delay when desired by removing the obturator. When withdrawing the arterial needle, be sure to apply pressure for a few minutes to prevent hematoma formation.

After the arterial needle is in place, insert a 19- or 20-gauge needle into one of the antecubital veins. Attach to the needle a water-tight, three-way stopcock; the side outlet of the stopcock is connected with a saline reservoir or a syringe filled with saline. Force saline gently into the vein until the obstruction to flow has disappeared. At this point collect a dye-free blood sample from the artery. Attach the syringe containing dye to the other outlet of the stopcock; inject the dye into the vein and record the time. Rinse the syringe two or three times by filling it with saline from the reservoir and expelling the contents into the vein. Make sure that all the dye is washed into the circulation. *The dye must not be injected into the artery.*

In normal subjects the average time required for complete mixing of the dye is about nine minutes. In cases of severe shock this is increased to fifteen minutes.²³ The ten-minute sample may therefore be supplemented by a fifteen-minute sample. However, the difference in the plasma volume calculated from these two will not in general exceed 4 to 5 per cent. If there is a difference, the fifteen-minute figure is taken as correct.

D. GENERAL CONSIDERATIONS IN THE MEASUREMENT OF BLOOD VOLUME

• Successful use of the dye method in measuring blood volume depends largely upon a knowledge of its limitations and the manner in which various conditions and technical errors may influence the results. The circumstances under which the measurement is made should, in every instance, be considered in regard to their possible effects on the accuracy of the plasma dye determination and on the mixing and disappearance of the dye.

*This chart is, of course, applicable only with the solution of T-1824 prepared and standardized for this purpose.

Determination of the Plasma Dye Concentration.—The first requirement for an accurate colorimetric determination of the plasma dye concentration is that the relation between the concentration and spectral absorption of the dye be fixed. The spectral absorption of T-1824 is influenced by salts, by the pH, and by colloids, but in plasma or serum the color of the dye is remarkably stable.¹⁹ This stability appears to be related to the phenomenon of selective binding of the dye by the plasma albumin.²³ The spectral absorption of T-1824 is the same over a very wide range of dye-albumin ratios. Nevertheless, *if the dye is used in cases presenting extreme pathologic changes in the composition of the blood, the dye solution should be standardized with plasma or serum from the patient in question.*

The second basic requirement is that the control (dye-free) and unknown (dye-tinged) plasma samples be identical in every respect except for the dye present in the latter. Otherwise the difference in optical density is not a true measure of the dye concentration. As will be seen, errors of this nature usually arise from failure to observe rather simple technical precautions.

Inherent Color of the Plasma.—The optical density of normal plasma at 620 to 625 m μ , the region of maximal absorption of T-1824 in plasma, is small (usually 0.1 or less) in comparison with the density (1.0 to 1.2) obtained after giving the usual amount of dye employed in determining the plasma volume. Consequently the effect of variations in the natural plasma color during the interval between the withdrawal of the control and unknown samples will, in general, be negligible. This is obviously no longer true if the optical density of the control plasma is high as, for example, when the plasma is already heavily tinged with dye remaining in the blood stream from previous injections (residual dye). It then becomes imperative to ascertain whether or not the plasma has changed in concentration during the interval in question. This information can best be obtained by comparing the plasma protein concentrations in the two samples, and, therefore, some convenient and rapid method of estimating the plasma proteins (refractometer, falling drop, or copper sulfate method) should always be included as a routine part of the procedure. If the hematocrit values and the plasma protein levels in the control and unknown do not agree, the reason is either (1) that the plasma volume is changing rapidly, or, more likely, (2) that the samples have been drawn without adequate precautions against stasis. Hence, when circumstances permit, it is advisable to draw duplicate samples separated by approximately a minute. The presence of stasis will be revealed by lower values for hematocrit and plasma protein in the second sample of such a pair, and when this is the case, the second sample should be taken for the determination.

It is evident from these considerations that for maximal accuracy in the photometric determination of the plasma dye concentration, (1) the samples must be obtained without stasis, and (2) the composition of the blood should remain fairly constant during the interval between the withdrawal of the control and unknown samples. This is especially true if for any reason (for example, residual dye, lipemia, hemolysis, high concentration of bile pigments) the optical density of the control plasma is large in comparison with the differ-

ence in density between the control and unknown. Hence it is not advisable to determine plasma volume while giving an infusion.*

The presence of lipemia may lead to considerable uncertainty in the dye determination. Whenever possible, the difficulty with lipemia should be avoided by planning the determination at a time when the patient is in the postabsorptive state. It may be stated however that in studies of accident cases, lipemia was seldom encountered.^{24, 29}

Theoretically, lipemia should not interfere with the dye determination if done with a spectrophotometer,¹⁶ provided the degree of lipemia is exactly the same in the control as in the unknown. Such may actually be the situation at the time of sampling. However, if the samples are allowed to stand for several hours, fat begins to accumulate at the surface of the sample (especially in the cold), and in the experience of the writer, the degree of lipemia may then vary considerably in a series of samples although taken simultaneously. The results appear to be more consistent if the dye determinations are done as soon as possible after the samples have been drawn. If the determination is delayed, some attempt should be made to extract most of the fat. This can be done either by centrifugation at high speed (18,000 r.p.m.) or by shaking with ethylene dichloride. Since the plasma is still somewhat opalescent after the treatment described, neither of these methods is completely satisfactory. More effective methods at present available for dealing with lipemia (extraction of the dye from the plasma,³⁰ or decoloration of the dye³¹) are fairly laborious and impractical for routine clinical or field determinations of blood volume. Furthermore, the reagents dilute the original plasma several fold, and hence it becomes necessary either to increase the sensitivity of the dye determination or to increase the dose of dye injected, which is undesirable because of the tissue staining that results.

Gross hemolysis inadvertently produced in the course of the drawing or subsequent handling of the blood samples is the commonest source of error in the dye determination. Although the errors from hemolysis are greatly minimized by using T-1824, which permits the readings to be made in the region of the spectrum above 600 m μ where the absorption by hemoglobin is relatively small,^{16, 19, 20} it must be emphasized that *marked hemolysis in either the control or the unknown sample may throw the determination off by 5 to 10 per cent.* The presence or absence of hemolysis should always be noted and recorded for each sample as evidence of whether or not the readings are trustworthy. The blue color of the dye does not obscure hemolysis. Even small traces of hemolysis in the plasma samples are readily detected by inspection of the tubes against a white background. If the degree of hemolysis is so slight that its presence is questionable, the observer may be assured that the effect on the dye determination will, for all practical purposes, be negligible.

Circumstances may be encountered in which the circulating plasma is already tinged with hemoglobin (severe burns, extensive contusions of muscles, and after transfusion with whole blood which has been stored for some time).

*Progressive bleeding, however, does not interfere with the measurement unless the bleeding is associated with rapid changes in the composition of the blood. To be sure, the blood that escapes during the ten-minute period allowed for mixing will contain dye but in approximately the same concentration as the blood remaining in the circulation. For this reason the calculated volume includes the blood lost during the mixing period. In other words, the determination will give the volume at the time of the dye injection rather than that in circulation when the dye-tinged sample is drawn.

This condition does not of itself interfere with the dye determination inasmuch as the hemoglobin content of the circulating plasma will be essentially the same during the short period of time required for a plasma volume determination.¹⁸ *The samples must, however, be obtained without additional hemolysis.* Unless the circulating plasma is definitely known to be tinged with hemoglobin, the presence of hemolysis in any of the plasma samples is invariably the result of failure to observe certain simple precautions:

1. All needles, syringes, sample tubes, and corks with which the blood comes into contact must be not only clean, but *absolutely dry*.

2. The cleaning of the equipment should be carefully supervised to make sure that soap or cleaning fluid used in the washing is completely removed by repeated rinsings.

3. Avoid drawing air into the syringe when collecting blood. When emptying the syringe, do not squirt the blood into the sample tube, but allow it to flow gently. Inclusion of air bubbles or froth in the sample is prone to cause hemolysis.

Mixing Time and Disappearance Rate of Dye.—The simplified procedure for measuring plasma volume outlined leads one to presume that the dye is uniformly mixed with the circulating plasma within ten minutes after the injection, and also that the amount of dye lost from the circulation during this interval is negligible. Are these assumptions valid? The evidence which is based on interpretations of the time-concentration curves will be considered briefly.

As Erlanger pointed out,¹ "Only that part of the (dye) concentration curve is indicative of the rate of disappearance which is inscribed after distribution (of dye) has become uniform." He also stated, "It is barely possible that by means of backward extrapolation from the latter parts of the curve it might be possible to determine not alone the percentage disappearance during the mixing period, but even the duration of the mixing period and the concentration at the termination of the injection." The writer adopted the extrapolation method of determining the initial dye concentration in the summer of 1934.

This procedure, which is an attempt to correct for the dye lost during the mixing period and, therefore, to make the determination of plasma volume independent of variations in mixing time as well as disappearance rate, has since been widely employed. In spite of the theoretical objections arising from the fact that the curve of disappearance of dye does not, strictly speaking, follow a straight line on a linear plot, the approximation is usually quite adequate for the purpose of measuring the volume. However, in all instances it cannot be relied upon to give a precise estimate of the time of mixing. Even in normal subjects at rest, one may occasionally find a time-concentration curve in which the "mixing" and "disappearance" portions of the curve blend so gradually that it is difficult to decide exactly at what point mixing ceases to influence the dye curve. Conditions involving slowing of the circulation (for example, shock) accentuate the difficulty. In all such instances the interpretation of the data necessarily depends upon the assumption which is made in regard to the exact form of the disappearance curve. Various lines of evidence

now available indicate that this may be defined by a straight line on a semilog plot (logarithm of the plasma dye concentration plotted against time). The evidence in part is as follows:

The remarkably slow rate of disappearance of T-1824³² and the effect of plasma or serum on its solubility and spectral absorption suggested the dye combines with plasma protein.¹⁹ This has recently been clearly demonstrated in various ways by Rawson,²⁸ who finds that T-1824 in plasma or serum is firmly and selectively bound to the albumin. Thus if the escape of T-1824 from the blood stream is determined mainly by the rate of exchange of albumin, the disappearance curve should be exponential in character.²¹ Careful examination of T-1824 time-concentration curves confirmed this prediction and showed that the disappearance of the dye is best defined by a straight line on a semilog plot.²¹ This conclusion removes the element of personal judgment from the estimation of the length of the mixing period. The latter is clearly revealed by the deviation of the early portion of the dye curve from the straight line.

It is apparent that any change in volume during the period of measurement may influence the dye curve and lead to an erroneous estimate not only of the mixing time, but also of the disappearance rate. If the plasma protein or hematocrit values obtained from the various dye-tinged blood samples indicate that such changes in volume have occurred, the dye readings must be adjusted by applying the factor P_o/P_t in which P_o is the plasma protein concentration in the control sample taken immediately prior to the injection of dye and P_t the protein concentration in the dye-tinged sample in which the observed dye reading is D_t . The corrected dye reading D_c is obtained by the following simple formula:

$$D_c = D_t \times \frac{P_o}{P_t}$$

In making this correction for fluid shifts, one obviously assumes that the total amount of circulating protein remains constant during the period covered by the dye curve. Determinations of plasma volume immediately before and after the occurrence of large fluid shifts demonstrate that the assumption, in general, is valid.

During the past year we have analyzed a large number of time-concentration curves to determine the mean values and range of variation in mixing time and disappearance rate of T-1824 in normal subjects and in patients suffering from various degrees of hemorrhage, skeletal trauma, abdominal injuries, head injuries, or burns.²⁵ In fifty-one normal male subjects the mean disappearance rate (expressed as per cent fall in dye concentration during the first hour after injection) was slightly over 5 per cent; the values ranged from 3 to 11 per cent per hour. The mean value for twenty-four patients in shock from hemorrhage or skeletal trauma was 8 per cent. In thirty-five patients with similar injuries but without symptoms of shock, the average disappearance rate was 8.4 per cent. In burns the dye escaped more rapidly, the average from six patients being about 15 per cent, with some values as high as 25 per cent. A similar increase in the disappearance rate was also found in certain cases of abdominal injuries. The average mixing time on normal subjects was nine minutes; in patients in shock, about fifteen minutes; after emergence from

shock, seven minutes. In patients with similar types of injuries but not in shock, the average mixing time was also about seven minutes.

These observations show clearly that there is little change in disappearance rate of dye with the appearance of symptoms of shock. A significant increase in the rate of escape of dye occurs only in patients in whom extensive areas of the capillary bed have been injured (for example, burns). Although in severe cases of shock there is some increase in the mixing time, it can by no means be claimed that mixing fails to occur. These data therefore refute the view that the dye method is unreliable for measuring plasma volume in shock. The conclusion that the dye method is reliable in this condition has also been reached by Evans and associates²⁹ from the examination of dye curves obtained on patients suffering from various degrees of shock.

Systematic analysis of the dye curves also demonstrated that a reliable determination of plasma volume can, as a rule, be made from the dye content of a single sample drawn ten minutes after the dye injection.²⁵ Comparison of the plasma volume calculated from the ten-minute sample with the more accurate results obtained by extrapolation of the disappearance curve to the time of injection showed only minor differences. Nearly all values calculated from the ten-minute sample fell between -2 and +4 per cent of the true plasma volume. It may be noted that any error arising from incomplete mixing tends to counterbalance the error introduced by disregarding the loss of dye during the mixing period.²⁵

The simplified technique of measuring plasma volume from a single dyed sample drawn at ten minutes must, of course, be used with discretion. Our data demonstrate that it is a valid procedure in normal subjects and in patients who have suffered severe injury from hemorrhage, trauma, burns, etc. Adequate data have not so far been obtained to permit general application of the technique to other types of circulatory failure (for example, cardiac failure) in which mixing may be greatly prolonged.

Estimation of Total Blood Volume from the Plasma Volume and Hematocrit.—The question here is whether or not the total blood volume as calculated from the formula $\frac{\text{Plasma volume c.c.}}{100 - \text{hematocrit } \%} \times 100$ corresponds to the true blood volume. This depends upon (a) the accuracy of the determination of the relative erythrocyte volume and (b) whether or not the sample selected for this purpose is truly representative of all the blood in the circulation.

(a) Comparison of the hematocrit method with other means of estimating the relative erythrocyte volume has shown that the former gives a value which is 3 to 4 per cent too high,* the reason being that a small fraction of the plasma remains trapped in the cell mass during centrifugation.^{22,34} Strictly speaking, a correction factor of 0.96 should therefore be applied to the hematocrit value as determined by centrifugation, or, if determined with the copper sulfate method, the figure used for specific gravity of the cells should be 1.1036 rather than 1.0971. However, for practical purposes this refinement is unnecessary, for in most cases the disturbance in blood volume must be gauged by comparison.

*Chapin and Ross³² find the difference to be about 8 per cent. Recently I again compared values obtained with T-1824 with those determined by centrifugation. In these experiments the difference in results with the two methods was again 3 to 4 per cent.

son with an assumed normal value derived from data which, in general, have not been corrected for the systematic error inherent in the hematocrit determination. Furthermore, the error is insignificant in comparison with the range of normal variations (see Fig. 7).

(b) The concentration of erythrocytes in the blood coursing through the large veins and arteries from which samples are customarily drawn is not necessarily the same as that in all parts of the vascular bed. In the spleen, for example, the per cent cells is higher,³³ whereas in the capillaries and small vessels the ratio of cells to plasma is considerably lower than that given by the central venous or arterial hematocrit.³⁰⁻³⁸ To what extent does this invalidate the calculation of total circulating red cell volume and total blood volume?

Measurements of blood volume with the carbon monoxide method by Smith and co-workers^{39, 40} and more recent observations with the radioactive iron method⁴¹ have indicated that the central venous hematocrit is considerably higher than the body hematocrit $\left(\frac{\text{total red cell volume}}{\text{total plasma volume} + \text{total red cell volume}} \right)$. That is to say, the total red cell volume calculated from plasma volume and the central hematocrit appears to be falsely high. Bazett and associates,⁴² however, found only slight differences in the total blood volumes determined with carbon monoxide and dye (congo red). Expressed as percentages of the real blood volumes (taken to be the sums of plasma and cells as separately determined), the average value obtained with the dye method was 103 per cent, and with the carbon monoxide, 96 per cent.

During the past year Roughton, Root, and the writer have re-investigated this question,¹⁸ employing improved methods for determination of carbon monoxide. In simultaneous measurements with the dye method (T-1824) and with carbon monoxide (inhalation of carbon monoxide or injection of blood saturated with carbon monoxide), the agreement is quite satisfactory. Expressed as per cent of the true volume (plasma volume determined with T-1824 plus red cell volume determined with carbon monoxide), the total blood volume calculated from plasma volume and the relative erythrocyte volume (centrifuge hematocrit $\times 0.96$) was as follows:

	AVERAGE
Normal male subjects	101
Normal dogs	99
Normal splenectomized dogs	103
Dogs in shock after muscle trauma	102
Dogs in shock after hemorrhage	97

It is clear from these observations that the total blood volume may be satisfactorily estimated from the plasma volume and the hematocrit not only under normal conditions, but also in conditions of shock. This has been indirectly confirmed by plasma volume studies on patients in shock.²⁵ In cases where bleeding had been stopped, the total red cell volume determined from the plasma volume and the hematocrit at the time of admission, plus the amount of cells given in transfusion, agreed closely with the total cell volume determined subsequently after the patient had emerged from shock.

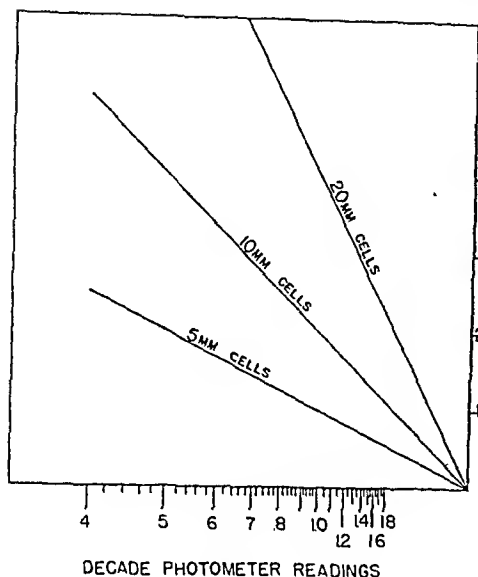


Fig. 5.

Fig. 5.—Chart for obtaining the plasma volume directly from the Decade Photometer reading (optical density of the ten-minute plasma sample read against the control plasma drawn just before the dye injection) when using the standard ampule of T-1824.

Fig. 6.—The relation of total plasma volume to body weight. The chart has been constructed from the average normal plasma volume per unit body weight (45 c.c./kg.). The broken lines indicate the range of normal variations.

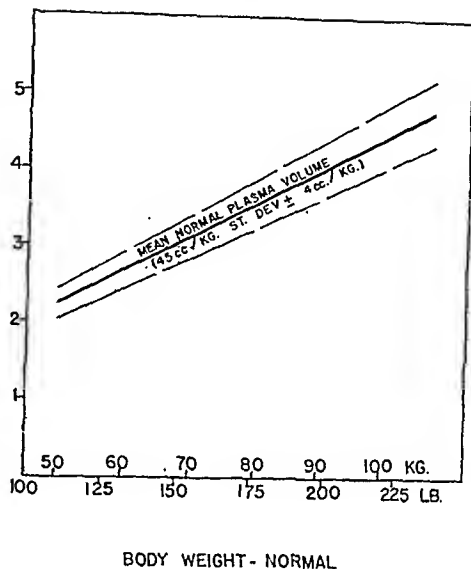


Fig. 6.

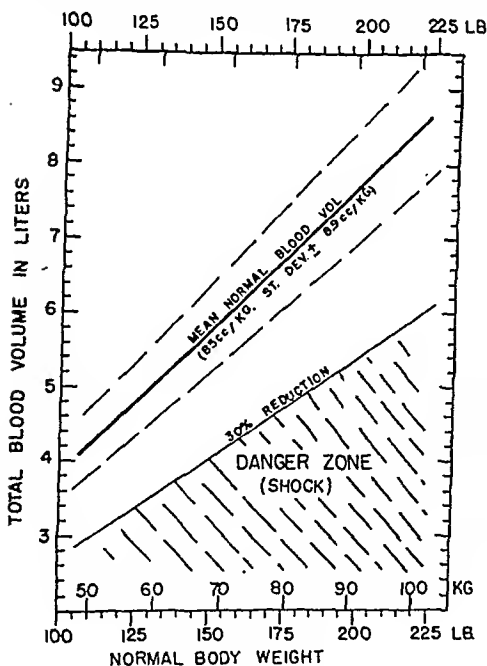


Fig. 7.

Fig. 7.—The relation of total blood volume to body weight. As in Fig. 6, the graph has been constructed from the average normal blood volume per unit body weight (85 cc./kg.). It will be seen that given this degree of reduction in volume, 2 or more liters of blood will be required to restore the blood volume to normal in a patient of average size.

Fig. 8.—Indicating approximately the normal amounts of total circulating hemoglobin, total red cell volume, and total circulating plasma protein in relation to weight.

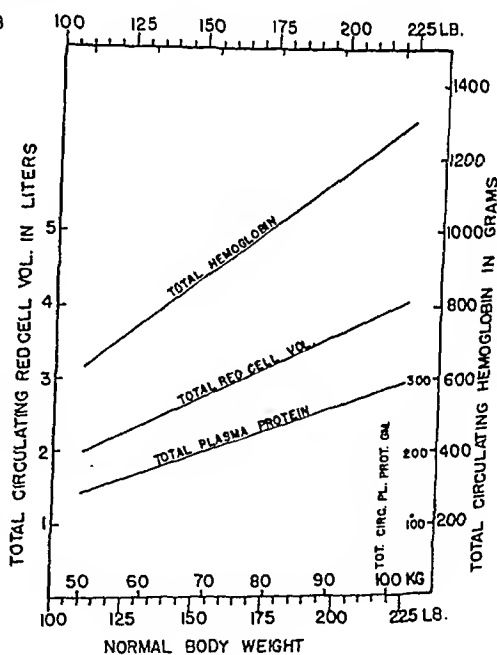


Fig. 8.

The foregoing facts must not be misconstrued. They do not disprove that the cell to plasma ratio is less in the capillaries than in the larger vessels. However, the extent to which such unequal distribution of red cells affects the estimation of total blood volume obviously depends on the fraction of the total blood present in the capillaries and small vessels. The blood held in this region of the vascular bed is estimated to be only 10 to 15 per cent of the total,⁴² and this is in agreement with the amount of plasma that appears to be involved in the slower phases of mixing.²¹

E. NORMAL BLOOD VOLUME IN MAN

Figs. 6 and 7 show the general relation of plasma volume and total blood volume to body weight. The charts were constructed by applying the average values obtained from measurements on normal young men to the entire range of body weights shown on the abscissa. The chart is therefore an approximation, but greater refinement does not seem justifiable until large series of measurements made with the same technique become available. Fig. 8 shows the relation of total circulating hemoglobin, red cell volume, and plasma protein to body weight; it has been constructed from the preceding blood volume charts and from average normal values for hemoglobin, per cent erythrocytes, and grams per cent plasma protein. Body weight rather than surface area has been chosen for the abscissae in Figs. 6, 7, and 8 for simplicity in utilizing the charts. As a matter of fact, the correlation of blood volume with body weight appears to be as satisfactory as with surface area. In emergencies where a patient cannot be weighed, his own report or an estimate of body weight by an experienced person may be quite satisfactory for purposes of determining the amount of blood or blood substitute required to bring the patient out of the danger zone.

DISCUSSION

In spite of the fact that reduction in blood volume has long been regarded as the primary cause of shock, with rare exception this measurement has been omitted from all but recent investigations of the problem. The general concepts of shock developed after World War I retarded the application of the dye method to this condition. It was believed that shock was accompanied by general increased capillary leakage of plasma, and hence, as pointed out above, it was thought that rapid escape of dye from the circulation would invalidate the results. However, the evidence obtained in this laboratory during the past three years^{18, 43} shows that the reduction in blood volume is fully accounted for without assuming that there is a generalized leakage of plasma from the circulation. Indeed, fluid is actually absorbed by the blood stream in the uninjured regions, and to a considerable extent this serves to compensate for the local loss of blood and fluid at the site of injury. It may be added that this is entirely in accord with the common finding that patients in shock after either hemorrhage or skeletal trauma show a tendency toward hemodilution rather than hemoconcentration.²⁵ The latter was observed only in patients who had suffered from exposure and dehydration subsequent to the injury or in patients where the injury involved a large area of the capillary bed (for example, burns, abdominal injuries with peritonitis). Although the disappearance

rate of dye may be considerably increased in cases of burns, the plasma volume can nevertheless be measured accurately by employing the extrapolation method which corrects for the dye lost during the mixing period. Even if the increased dye loss is disregarded and the volume is determined from a single dye-tinged sample taken at ten minutes after the injection, the error in measurement is only 3 to 4 per cent.

The radical changes in distribution and flow of blood in severe shock require that careful consideration always be given to the matter of obtaining a "fair" sample of the circulating blood. Peripheral vasoconstriction in shock ordinarily precludes the use of superficial veins, and the samples must therefore be drawn from deeper veins or preferably from an artery. Failure to observe this precaution has probably been one of the principal reasons why some investigators have been unable to obtain consistent results with the dye method. The precaution of course applies equally to other methods of determining blood volume.

The potential value of blood volume determinations in the diagnosis, prognosis, and treatment of shock has been greatly enhanced by recent experimental studies on animals¹⁸ as well as by studies on human cases of shock.^{24, 25} The results of these investigations fully confirm the general conclusions reached by Keith⁴⁴ and Robertson and Bock⁴⁵ from their studies on wounded men during World War I. In cases of hemorrhage and skeletal trauma, the train of events leading to shock is initiated by a large reduction in blood volume.^{18, 43} In both dog and man an acute reduction of 30 to 40 per cent in the circulating blood volume is followed within one or two hours by the appearance of characteristic symptoms of shock. For practical purposes of therapy, it may therefore be concluded that a patient showing the clinical picture of severe shock after hemorrhage or skeletal trauma has lost about two quarts of blood.

Since the reduction in blood volume precedes the appearance of the symptoms of shock, it constitutes a valuable prodromal sign, and early determination of blood volume may therefore be most useful as a means of predicting whether or not shock is impending. With this information available, shock can be combatted before it develops. Furthermore, one can estimate the minimum amount of blood or blood substitute necessary to bring a patient out of danger of shock from reduced blood volume (see Fig. 7). It is obvious that this may be a matter of critical importance where only a limited supply of blood or blood substitute happens to be available for a large number of casualties.

Ideally, intravenous therapy should achieve restoration of the normal volume as well as the normal composition of the blood. For lack of better criteria the doctor often depends upon hematocrit and plasma protein measurements as indexes of whether or not adequate amounts of blood or plasma have been given. These data may be misleading, however, especially in cases where variable amounts of cells and plasma proteins are being continuously lost from the circulation (undisclosed slow hemorrhage, burns, abdominal wounds, peritonitis). The danger lies not only in failure to maintain an adequate blood volume, but also in the excessive use of plasma, etc., and thereby running the risk of inducing heart failure or pulmonary edema. In these cases, a determination of the blood volume provides a reliable guide to therapy.

Great emphasis has been placed on the role of diminished blood volume in causing shock. One must not, however, lose sight of the fact that failure of the circulation may occur without any essential change in the amount of circulating blood. This may be the case in head injuries,^{24, 25} in bleeding into the pericardium (cardiac tamponade), myocardial failure, and in toxemias similar to histamine shock.⁴⁶ In such cases a determination of blood volume is an aid in the differential diagnosis and shows whether or not transfusion should be included in the treatment.

Another important use for blood volume determinations has appeared in the study of convalescent patients and those with chronic wound infections.⁴⁷ In these patients, who usually undergo extensive loss in body weight over a period of weeks or months, the deficiency in blood volume should probably be evaluated in terms of the individual's normal body weight. On this basis, the reduction in volume may be of the order of 60 to 70 per cent.⁴⁸ Nevertheless, the hematocrit value and the plasma protein concentration may remain essentially normal and give no indication of the large deficiency in blood volume.

Finally, it is well to note that the present emergency provides an unparalleled opportunity for determining normal standards of blood volume on comparable groups of men living under a great variety of environmental conditions. Indeed, such normal standards are essential for the correct interpretation of blood volume on casualties in the various theaters of war. A broad study of blood volume in men exposed to arctic, desert, and tropical environments should also be undertaken with the aim of determining the blood volume levels required for maximal physiologic efficiency.

The technique described has been successfully employed for some months by Major Champ Lyons, Major John Stewart, and Captain Oeta Leigh, who undertook to make practical tests of the method in the European theater of operations.

SUMMARY

A practical method of determining plasma volume and total blood volume, designed for use by the Armed Forces, is described. The method is based upon measurement of plasma volume with the dye T-1824. The total blood volume is then calculated from the plasma volume and the hematocrit. All of the equipment is portable.

Simplification of the technique is dependent on (a) the use of the new Decade Photometer recently designed for this purpose by Nickerson;²⁶ (b) the demonstration from analysis of a large series of time-concentration curves of T-1824 that a single dye-tinged sample taken at ten minutes after injection of dye is adequate for a reliable determination of the plasma volume; (c) the use of a standard amount of dye for the determinations with the result that the plasma volume is obtained directly from the photometer reading; (d) the use of the copper sulfate-specific gravity method of Phillips and co-workers²⁷ instead of a centrifuge for the determination of the relative erythrocyte volume (hematocrit).

The precautions which are necessary for successful use of the present method and also various theoretical considerations pertaining to the measurement of blood volume are discussed in some detail.

Some of the practical applications of blood volume determinations to the problems of shock, burns, convalescence, physiologic efficiency, etc., are mentioned briefly.

APPENDIX

Calculation of Plasma Volume.—The plasma volume is calculated from the relation $C_1 \times V_1 = C_2 \times V_2$, where

C_1 = concentration of dye solution injected

V_1 = c.c. of dye injected

C_2 = concentration of dye in plasma obtained ten minutes after the injection

V_2 = plasma volume.

Since the concentration of dye is directly proportional to the optical density, the equation may be written $(D_1 \times 500) V_1 = D_2 \times V_2$ in which

D_1 = the density of the standard dye solution diluted 1:500 in plasma, and

D_2 = the density of dye in the circulating plasma after uniform mixing (ten minutes). Hence, plasma volume in c.c. = $\frac{D_1 \times 500 \times V_1}{D_2}$

The concentration of dye in the standard ampule (0.46 per cent) has been adjusted to give a value for D_1 of 0.8 when using 10 mm. absorption cells. Since V_1 is also a constant (5 c.c.) and D_2 is read directly on the photometer

Plasma volume in c.c. = $\frac{0.8 \times 500 \times 5}{D_2}$ or simply $\frac{2,000}{\text{Photometer reading}}$

A wide range of plasma volumes (1,150 to 6,500) can be determined without any change in the procedure outlined. If, however, the photometer reading exceeds 1.79 (plasma volume less than 1,150), read the sample in 5 mm. depth instead of 10, multiply the reading by 2, and proceed as before in calculating the plasma volume. If the reading approaches 0.3 (plasma volume greater than 6,500), read the sample in 15 mm. depth and divide the value by 1.5. In this manner the range may be extended to include plasma volumes from 600 c.c. up to 10,000 without altering the amount of dye injected (see Fig. 5).

The dose of T-1824 in the standard 5 c.c. ampule is adjusted for determinations on adults. Unless given several times within a period of a few hours, this amount (approximately 23 mg.) will not cause visible staining of the subject. For routine determinations on small subjects or on children it is advisable to reduce the dose. This may be done without sacrificing accuracy by reducing the concentration of the dye solution rather than the volume injected.

Standardizing the Dye.—Use only pipettes and flasks that have been carefully calibrated.

A. 1:500 dilution of standard dye solution in distilled water. Deliver exactly 1 c.c. of dye from the standard ampule (0.46 per cent) into a 50 c.c. volumetric flask and make up to volume with distilled water.

B. 1:500 dilution of dye in plasma. Mix 0.2 c.c. of A with 1.8 c.c. of clear plasma or serum.

C. Control. Mix 0.2 c.c. of distilled water with 1.8 c.c. of the same plasma used in preparing B.

Read B against A in the Decade Photometer, using 10 mm. absorption cells. The value obtained is D_1 in the equation given.

REFERENCES

1. Erlanger, J.: Blood Volume and Its Regulation, *Physiol. Rev.* 1: 177, 1921.
2. von Behring, E.: Antitoxin for Plasma Determinations, *Beitr. z. Exper. Ther.* 12: 2, 1912.
3. Culbertson, J. T.: The Determination of the Plasma Volume and the Blood Volume of the Rabbit by the Injection of Homologous Anti-Crystallized-Egg-Albumin-Serum, *Am. J. Physiol.* 107: 120, 1934.
4. Madsen, E.: En Metode Til Bestemmelse Af Menneskets Blodmaengde Ved Hjaelp Af Differancieltoksin, Copenhagen, 1936.
5. Keith, N. M., Rowntree, L. G., and Geraghty, J. T.: A Method for the Determination of Plasma and Blood Volume, *Arch. Int. Med.* 16: 547, 1915.
6. Meek, W. J., and Gasser, H. S.: Blood Volume: A Method for Its Determination With Data for Dogs, Cats and Rabbits, *Am. J. Physiol.* 47: 302, 1918-1919.
7. Barratt, J. O. W., and Yorke, W.: A Method of Estimating the Total Volume of Blood Contained in the Living Body, *Roy. Soc. London Proc.* 81: 381, 1909.
8. Lee, F. W., and Whipple, G. H.: Blood Volume Studies; Plasma Volume as Determined by Hemoglobin Injection, *Am. J. Physiol.* 56: 328, 1921.
9. Haldane, J., and Smith, J. L.: The Mass and Oxygen Capacity of the Blood in Man, *J. Physiol.* 25: 331, 1899.
10. Grehant, N., and Quinquand, E.: Mesure du Volume de Sang Contenu dans l'organisme d'un Mammifere Vivant, *Compt. rend. Acad. d. sc., Par.* 94: 1450, 1882.
11. Hahn, P. F., Ross, J. F., Bale, W. F., Balfour, W. M., and Whipple, G. H.: Red Cell and Plasma Volumes (Circulating and Total) as Determined by Radio Iron and by Dye, *J. Exper. Med.* 75: 221, 1942.
12. Hahn, L., and Hevesy, G.: Radioactive Phosphorous for Red Cell Volume Determinations, *Acta Physiol. Scand.* 1: 3, 1940.
13. Anderson, R. S.: The Use of Radioactive Phosphorous for Determining Circulating Erythrocyte Volumes, *Am. J. Physiol.* 137: 539, 1942.
14. Magladery, J. W., Solandt, D. Y., and Best, C. H.: Serum and Plasma in Treatment of Haemorrhage in Experimental Animals, *Brit. M. J.* 2: 248, 1940.
15. Freeman, N. E.: Burns, Shock, Wound Healing and Vascular Injuries, *Military Surgical Manual*, National Research Council, Philadelphia, 1943, W. B. Saunders Co., p. 107.
16. Gregersen, M. I.: An Analysis of Colorimetric Methods in Relation to Plasma Volume Determinations, *J. LAB. & CLIN. MED.* 23: 423, 1938.
17. Gregersen, M. I.: Macleod's Physiology in Modern Medicine, ed. 9, St. Louis, 1941, The C. V. Mosby Co., p. 1,067.
18. Gregersen, M. I., and others: Unpublished reports.
19. Gregersen, M. I., and Gibson, J. G.: Conditions Affecting the Absorption Spectra of Vital Dyes in Plasma, *Am. J. Physiol.* 120: 494, 1937.
20. Gregersen, M. I., Gibson, J. G., and Stead, E. A.: Plasma Volume Determination With Dyes: Errors in Colorimetry; the Use of the Blue Dye T-1824, *Am. J. Physiol.* 113: 54, 1935.
21. Gregersen, M. I., and Rawson, R. A.: The Disappearance of T-1824 and Structurally Related Dyes from the Blood Stream, *Am. J. Physiol.* 138: 698, 1943.
22. Gregersen, M. I., and Sehiro, H.: The Behavior of the Dye T-1824 With Respect to Its Absorption by Red Cells and Its Fate in the Blood Undergoing Coagulation, *Am. J. Physiol.* 121: 284, 1938.
23. Gregersen, M. I., and Stewart, J. D.: Simultaneous Determination of the Plasma Volume With T-1824 and the "Available Fluid" Volume with Sodium Thiocyanate, *Am. J. Physiol.* 125: 142, 1939.
24. Courmand, A., Riley, R. L., Bradley, S. E., Breed, E. S., Noble, R. P., Lauson, H. D., Gregersen, M. I., and Richards, D. W.: Studies of Circulation in Clinical Shock, *Surgery* 13: 964, 1943.
25. Noble, R. P., and Gregersen, M. I.: To be published.
26. Nickerson, J. L.: A Decade Photometer for the Determination of Blood Volume With the Dye T-1824, *Rev. Scient. Instruments* 15: 69, 1944.
27. Phillips, R. A., Van Slyke, D. D., Dole, V. P., Emerson, K. Jr., Hamilton, P. B., and Archibald, R. M.: Copper Sulfate Method for Measuring Specific Gravities of Whole Blood and Plasma, *Bull. U. S. Army M. Dept. No. 71*, p. 66, 1943.
28. Rawson, R. A.: The Binding of T-1824 and Structurally Related Diazo Dyes by the Plasma Proteins, *Am. Jour. Physiol.* 138: 708 (April) 1943.
29. Evans, E. I.: Personal communication; Evans, E. I., Hoover, M. J., James, G. W., and Alm, T.: Studies on Traumatic Shock: I. Blood Volume Changes in Traumatic Shock, *Ann. Surg.* 119: 64, 1944.
30. Crooke, A. C., and Morris, C. J. A.: The Determination of Plasma Volume by the Evans Blue Method, *J. Physiol.* 101: 217, 1942.
31. Phillips, R. A.: A Method for the Determination of the Blue Dye T-1824 in Plasma, *J. Exper. Med.* 77: 421, 1943.
32. Dawson, A. B., Evans, H. M., and Whipple, G. H.: Blood Volume Studies. Behavior of Large Series of Dyes Introduced Into the Circulating Blood, *Am. J. Physiol.* 51: 232, 1920.

33. Chapin, M. A., and Ross, J. F.: The Determination of the True Cell Volume by Dye Dilution and With Radioactive Iron. The Error of the Centrifuge Hematocrit, *Am. J. Physiol.* 137: 447, 1942.
34. Shohl, A., and Hunter, T.: The Measurement of Cell Volume of Blood by the Evans Blue Dye Method, *J. LAB. & CLIN. MED.* 26: 1829, 1941.
35. Barcroft, J.: *Features in the Architecture of Physiology Functions*, ed. 1, London, 1934, Cambridge University Press.
36. Fahraeus, R.: The Suspension Stability of the Blood, *Physiol. Rev.* 9: 241, 1929.
37. Fahraeus, R., and Lindqvist, T.: The Viscosity of the Blood in Narrow Capillary Tubes, *Am. J. Physiol.* 96: 562, 1931.
38. Ebert, R. V., and Stead, E. A.: Demonstration That the Cell Plasma Ratio of Blood Contained in Minute Vessels Is Lower Than That of Venous Blood, *J. Clin. Investigation* 20: 317, 1941.
39. Smith, H. P., Arnold, H. R., and Whipple, G. H.: Blood Volume Studies. VII. Comparative Values of Welcker Carbon Monoxide and Dye Methods for Blood Volume Determinations, *Am. J. Physiol.* 56: 336, 1921.
40. Smith, H. P., Belt, A. E., Arnold, H. R., and Carrier, E. B.: Blood Volume Changes at High Altitude, *Am. J. Physiol.* 71: 395, 1924.
41. Bazett, H. C., Sunderman, F. W., Maxfield, M. E., and Scott, J. C.: Comparison of Estimates of Blood Volume Made by Congo Red and by Carbon Monoxide, *Am. J. Physiol.* 129: 309, 1940.
42. Bazett, H. C.: *Macleod's Physiology in Modern Medicine*, ed. 9, St. Louis, 1941, The C. V. Mosby Co., p. 341.
43. Gregersen, M. I.: Traumatic Shock, *Bull. New York Acad. Med.* 19: 666, 1943.
44. Keith, N. M.: Med. Research Committee Special Report, No. 27, 1919.
45. Robertson, O. H., and Bock, A. V.: Blood Volume in Wounded Soldiers. I. Blood Volume and Related Blood Changes After Hemorrhage, *J. Exper. Med.* 29: 139, 1919.
46. Deyrup, I. J.: Circulatory Changes Following the Subcutaneous Injection of Histamine in Dogs, *Am. J. Physiology* 142: 158, 1944.
47. Lyons, C.: Penicillin Therapy of Surgical Infections in the U. S. Army, *J. A. M. A.* 123: 1007, 1943.
48. Gregersen, M. I.: Unpublished observations.

LABORATORY METHODS

GENERAL

SUITABILITY OF SERUM FROM RECALCIFIED HUMAN PLASMA FOR SOME SERODIAGNOSTIC TESTS FOR SYPHILIS

CAPTAIN ROBERT D. BARNARD
MEDICAL CORPS, ARMY OF THE UNITED STATES

WITH THE COLLABORATION OF
MAJOR CHARLES R. REIN
MEDICAL CORPS, ARMY OF THE UNITED STATES

THE suitability of human plasma for the serologic tests for syphilis has received only desultory attention. Serum has, in the past, been almost universally used for all forms of these tests, except for those based on coagulation. With the introduction of large scale plasma banks, it became the practice, when the accompanying pilot sample of serum was lost, to use a small portion of the plasma unit instead. While Laughlen mentions the suitability of plasma for his "agglutination" test¹ and others have employed oxalated and fluorided plasma when these only were available,² no extensive study of the validity of this departure has appeared. Certain objections to it, however, might be raised: (1) the anticomplementary activity of citrate³ (evidenced only when complement fixation is to be attempted), (2) the flocculation of antigen which increases with citrate concentration,⁴ and (3) the turbidity of plasma which contrasts with the relative clarity of its corresponding serum. Plasma is clarified with difficulty even when prolonged centrifugation is done, and flocculation may be obscured by the presence in the field of amorphous, stably suspended material. In addition to these practical objections to the use of plasma for serodiagnostic tests, the presence of fibrinogen is an uncontrolled factor in the regulation of flocculation. The procedures have been adjusted so as to provide optimum conditions when serum is used. For the last reason many investigators have attempted the recalcification of plasma to remove the fibrinogen. Such recalcification, however, raises the calcium level of the serum sample to a concentration far above that obtained physiologically. The material must also be heated or alternately frozen and thawed to initiate the precipitation of fibrin. If the final concentration of calcium is less than five times the normal physiologic amount, fibrin formation is incomplete and takes the form of a gelatinization from which the subsequent separation of serum is difficult.

This work was prosecuted for the most part in the Division of Medical Research, the Department of Medicine, Ohio State University, Columbus, Ohio.

Received for publication, Sept. 20, 1944.

It was found that recalcified citrated plasma, made by adding a soluble calcium salt (calcium chloride) to plasma and alternately freezing and thawing it after a preliminary incubation period of from twelve to twenty-four hours, could be used for serologic testing with what appeared to be consistent results on both positively and negatively reacting serums (Table I). The method, however, had other objections in addition to its laboriousness. There was an extreme variability in the amount of calcium chloride solution required to effect fibrin formation. This observation was not a new one on our part; in the prothrombin time evaluation methods, it is customary to add various amounts of calcium salt through a range sufficient to give a final concentration of from 40 to 125 mg. per 100 c.c. In fact, the difficulty of causing clot formation in well-clarified plasma by any concentration of calcium whatever in the absence of thromboplastic substances forms the basis of the conglutination tests. The concentration of soluble calcium salt necessary for the conversion

TABLE I
COMPARATIVE SEROLOGIC RESULTS ON SERUM AND RECALCIFIED* PLASMA

CASE	SAMPLE	KD	KE	B-J-L	M	KN	KO
O. L.	S†				Pos.	Pos.	
	RCCP	4	4	4	4	3 4 4	AC
J. P.	S				Pos.	Pos.	
	RCCP	4	4	4	4	0 3 3	4
M. R.	S				Pos.	Pos.	
	RCCP	4	4	4	4	4 4 4	4
C. V.	S				Pos.	Pos.	
	RCCP	4	4	4	4	4 4 4	4
M. V.	S	-	±	-	-	0 0 0	-
	RCCP	-	±	-	-	0 0 0	-
H. B. J.	S	-	-	h	-	0 0 0	-
	RCCP	-	-	-	-	0 0 0	-
V. B.	S	±	±	±	-	0 0 0	-
	RCCP	±	±	±	-	0 0 0	-
A. T.	S				Pos.	Neg.	
	RCCP	1	4	2	3		2
M. R.	S	1	2, 3	1	±		AC
	RCCP	3	4	2	3	4 4 4	4
A. B.	S				Pos.	Neg.	
	RCCP	2	4	4	4	0 1 1	AC
H. P.	S				Pos.	Pos.	
	RCCP	4	4	4	4	4 4 4	4
T. P. B.	S				Pos.	Pos.	
	RCCP	4	4	4	4	4 4 4	3

*CaCl₂ added, 0.9 mg. per cubic centimeter of plasma which was then alternately frozen and thawed. The minimal concentration of CaCl₂ in buffered saline found to cause flocculation of the KD antigen was 0.15 per cent.

†1. Materials subjected to serologic testing (samples):

- S, Serum obtained from spontaneously clotted blood.
- SRCP, Serum obtained from recalcified (CaHPO₄) citrated plasma.
- SRGP, Serum obtained from recalcified glycerophosphated plasma.
- CP, Citrated plasma.
- GP, Glycerophosphated plasma.
- FP, Fluorided plasma.
- RFP, Recalcified fluorided plasma (no clot obtained).
- RSP, Recalcified saccharined plasma (no clot obtained).
- RCP, Recalcified (with Ca₂(PO₄)₂) citrated plasma (incomplete clotting).
- RCCP, CaCl₂ recalcified plasma.

2. Degree of positivity:

- a, Atypical positive reaction.
- sh, Slightly heavy negative (minimal flocculation).
- h, Heavy, negative.
- , negative reaction.
- 1, 2, 3, 4, degrees of positivity: 4, 8, 16, unitage of complete positivity.
- Pos., neg., doubtful, So reported by independent testing laboratory.
- , 0, Negative.
- AC, anticomplementary.

3. Type of test employed:

- KE, Kline exclusion
- KD, Kline diagnostic
- B-J-L, Boerner-Jones-Lukens

- KN, Kahn (three-tube standard).
- M, Mazzini.
- KO, Kolmer complement fixation.

of all samples of plasma to serum may interfere with certain flocculation tests either by an artificial flocculation of the antigen (thus giving rise to false positive reactions) or by peptization and dispersion of the antigen-reagin floc-cules (false negative reactions).

For these reasons, attempts were made to develop a recalcification procedure which would not involve an inordinate rise in the resulting serum calcium concentration. The accelerating effect of foreign particulate matter on coagulation was found to be especially pronounced when this matter was in the form of relatively insoluble calcium salts. A method was devised, therefore, which is based on the use of relatively insoluble calcium salts for the rapid recalcification of plasma.

The experimental study of the method was restricted to trial of those weakly soluble calcium salts, the anions of which were present physiologically. Secondary calcium phosphate (dicalcium phosphate), hydrated calcium sulfate (gypsum), calcium carbonate (chalk), tertiary calcium phosphate, and calcium magnesium inositol hexa-phosphate (phytin), when added to citrated plasma in excess of their solubility, caused fibrin formation. The salts are listed in the order of their activity. Adult beef bone meal did not induce coagulation.

Only the first and third salts induced sufficiently rapid coagulation for the purpose at hand. Calcium carbonate is somewhat more satisfactory for the conversion of oxalated plasma to serum, but the occasion for this conversion seldom arises. For conversion of citrated plasma, secondary calcium phosphate is the calcifier of choice. The efficacy of a particularly weak calcium salt in producing coagulation of citrated plasma is not in proportion to its solubility. Thus, gypsum and chalk are much more soluble than secondary calcium phosphate, yet the last is the most efficient calcifier we have been able to find. The dissolved calcium fraction from any of the salts used does not approach that concentration found necessary when coagulation is initiated by the addition of calcium chloride. These facts are in keeping with the theories of Ferguson,⁵ who was inclined to discount any intrinsic role of the calcium ion in the coagulation process.

Among all the calcium salts studied in this connection, dicalcium phosphate is unique in that its use is frequently followed by complete clot retraction in a time sequence and manner which appears to parallel that of the normal process.

We have investigated the validity of the ordinary serologic tests for syphilis when the latter are conducted on such artificially produced serum and its antecedent plasma. The study indicates that this expedient may actually be a more desirable routine than the now practiced one of performing serologic tests on unmodified serum, for when citrated blood is collected for these tests, there are certain advantages. Among these may be cited the greater resistance to hemolysis of citrated blood specimens, the ease of separating the cells without centrifugation soon after collection, the elimination of many doubtful reactions, and the greater retention of reagin titer on storage of citrated plasma.

EXPERIMENTAL

1. *Collection of Samples for Serologic Study.*—The smaller samples of blood were obtained by venipuncture with a 20 c.c. glass syringe and 20 caliber needle. The blood was withdrawn rapidly and 10 c.c. were immediately transferred to a

test tube containing 1 c.c. of 2.5 per cent sodium citrate. (In certain cases, 3.8 per cent citrate was used.) The second 10 c.c. portion of blood was similarly emptied into another tube containing 1 c.c. of 0.9 per cent sodium chloride. Thus, the citrated plasma and serum samples were of equal dilution with regard to the reagin and initial calcium concentrations. Larger plasma samples of approximately the same citrate concentration (500 c.c. of blood plus 50 c.c. of 2.5 per cent sodium citrate) were obtained.* This plasma was discarded from blood banks either because of seropositivity (whether connoting syphilis or not) or because of hemolysis or lipemia. Occasional samples of glycerophosphated, fluorided, and saccharinized plasma were also collected.

2. Recalcification With $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$.—Either citrated or glycerophosphated whole blood or plasma was converted to serum by adding 0.2 Gm. of secondary calcium phosphate to each 10 c.c. of the liquid. The process appeared to take no longer than does physiologic coagulation. Where plasma was the material used, the sequence of this induced coagulation was followed. It could be divided into four or more less distinct phases:

First phase. The secondary calcium phosphate particles were suspended discretely on agitation or inversion.

Second phase. After from seven to twelve minutes, the powdered secondary calcium phosphate had been aggregated into a button which, when broken up by vigorous agitation, showed many coalescent coarse particles.

Third phase. In about ten to twenty minutes after the addition of the secondary calcium phosphate, filar fibrin strands made their appearance in the body of the liquid. Following this preliminary appearance of the filar fibrin, the liquid became viscous and gelatinized throughout.

Fourth phase. The jelly retracted from the sides and bottom of the test tube, either completely in the case of fresh plasma or partially in the case of old plasma. In old plasma, there had already been considerable precipitation of filar fibrin without the addition of calcium, and the relative absence of clot retraction in aged plasma indicates that the filar form of fibrin is probably that concerned with retraction. But even in month-old plasma there was usually sufficient retraction after recalcification so that the clot floated in the mother liquid. The fourth phase of coagulation of recalcified plasma was absent if some other calcium salt was substituted for the secondary phosphate.

3. Transportation of Specimens.—Samples for serologic testing were most frequently collected at a place remote from the laboratory where the serologic testing was done. A large proportion of these samples were sent through the mail or in some other manner so that there was considerable delay before the tests were done. The receipt of specimens unsuitable for serologic testing by laboratories located in the same city where the specimen had been collected was so frequent an occurrence as to constitute a definite economic waste. Specimens became unsuitable during the period which elapsed after the collection because of (A) *hemolysis*, which prevented reading of flocculation or complement fixation; (B) *bacterial contamination*, which induced anticomplementary reactions and, through turbidity, obscured the reading of flocculation; and (C) *loss of reagin titer*. This had been indicated for the pseudoseropositivity following

*From Eli Lilly and Co.

vaccinia,⁶ and we have observed it in a number of instances in syphilitic seropositive samples, in the latter instance accompanied by the formation of a precipitate (autoflocculation).

A. Hemolysis: Where a long delay between collection and testing was anticipated, the effect of hemolysis was usually circumvented by removing the serum from the clot and forwarding the former to the testing laboratory. If a centrifuge was not available, we waited for several hours until retraction had occurred. Even then, due to the irregularity of the clot, serum recovery was low. Good plasma recovery, however, was possible within fifteen minutes of collection and citratization as in the procedure just described. Sedimentation of erythrocytes from .25 per cent citrated plasma was rapid without the necessity of any centrifugation, and the supernatant plasma was transferred to another tube by means of a medicine dropper. However, such a separation was not necessary, for it has been observed that in citrated blood overt hemolysis does not occur for days after collection and, when it does appear, is not nearly so marked as in a corresponding sample of whole clotted blood. When comparative samples were shipped together from the Midwest to the East, the clotted blood not infrequently was grossly hemolyzed; the citrated blood rarely showed any hemolysis whatsoever.

B. Bacterial contamination: Bacterial contamination was limited by the addition of a small amount of merthiolate, sulfonamide, or succinimide, none of which appeared to interfere with the conduct of the serologic tests. Aside from a few experiments on fluorided specimens (later abandoned because of the impossibility of securing good clots on recalcification), nothing was done on the problem of spoilage through bacterial action. It was noticed, however, that where comparative samples of citrated plasma and serum were mailed simultaneously, the bacterial spoilage apparent on arrival at the point of testing was much less in the case of plasma than in that of serum, even though no special precautions were taken to protect either type of sample.

C. Loss of Reagin Titer: In time the nature of the loss of titer of seropositive samples will be the subject of a report. The loss of titer was not evidenced, at least to the same degree, in citrated plasma as in serum. It is believed that the loss of titer of seropositive serum begins at the moment of coagulation and is due to an "auto-antigen" action, whereby the reagin is flocculated from solution prior to the conduct of the test. This autoflocculation apparently does not occur in specimens in which coagulation has not taken place, nor, for some reason not clearly understood, does it occur in the specimens recalcified as described. Whatever the theoretic connotations of the last, its practical importance lies in the fact that recalcified citrated specimens are greatly superior in this respect also to serum specimens, because the former, at least in the case of true syphilitic reactions, seem to have a higher titer.

SEROLOGIC TESTING OF SPECIMENS

The results of serologic testing of serum, citrated plasma, and recalcified specimens reported in this paper are derived from two sources:

1. In the case of plasma from whole blood units, the report on the accompanying pilot specimen of serum was obtained from the processing center; this report was given as "positive" or "negative" without quantitative connotation.

or intermediate degree. It was learned that the positive designation implied, on the basis of the three-tube Kahn technique, any reaction exceeding the arbitrary 2 plus; anything less was termed negative. Where a dubious or doubtful Kahn reaction had been encountered, the results of the Mazzini test (which had been run as a preliminary presumptive test to screen out those units to be subjected to further study) were available in terms of degree of positivity. The Mazzini test used as a screen procedure by the processing center was apparently more sensitive than the Mazzini employed in the battery, later described, for a 3 plus report in the former instance usually corresponded to between a 1 plus and 2 plus for the latter.

TABLE II

COMPARATIVE SEROLOGIC RESULTS ON SERUM OBTAINED FROM SPONTANEOUSLY CLOTTED BLOOD AND FROM PLASMA TREATED WITH CALCIUM PHOSPHATE

CASE	SAMPLE	KD	KE	B-J-L	M	KN	KO	CA*
D. D.	S†	-	sh	-	-	-	-	8.5
	SRCP	-	-	-	-	-	-	7.7
J. H.	SRCP	-	-	-	-	-	-	9.1
	S	-	-	-	-	-	-	
A. P.	SRCP	-	sh	-	-	-	-	
	S	-	-	-	-	-	-	
G. D.	SRCP	-	sh	-	-	-	-	9.8
	S	-	-	-	-	-	-	
M. R.	SRCP	-	sh	-	-	-	-	9.0
	S	-	-	-	-	-	-	7.9
E. H.	SRCP	-	sh	-	-	-	-	
	S	-	-	-	-	-	-	
D. R.	SRCP	1	2	1	-	-	-	10.3
	S	1	2	1	-	-	-	8.4
R. H.	SRCP	sh	±	±	sh	-	-	9.9
	S	sh	±	sh	-	-	-	
D. C.	SRCP	sh	±	±	sh	-	-	
	S	sh	±	±	sh	-	-	
R. L.	SRCP	-	-	-	-	-	-	9.2
M. T.	SRCP	32	128	32	64	4 4 2	4	16.8
	S	32	-	32	64	4 3 1	4	10.4
G. P.	SRCP	16	64	16	32	0 0 3	4	
	S	4	4	4	8	-	-	
H. V. II.	SRCP	8	32	8	32	1 2 ±	4	
	S	8	4	4	32	0 2 1	4	
	RCCP	16	64	32	32	4 4 4	4	
L. W.	S	4	4	3	4	-	0	
	SRCP	1	3	2	4	-	0	
B. Bo.	S	-	-	-	-	-	-	5.1(?)
	GP	±	1	±	1	-	-	
	SRCP	±	1	±	1	-	-	9.4
Ath.	S	4	4	4	4	4 4 4	4	
	FP	4	4	4	4	4 4 3	4	
	RFP	4	4	4	4	0 4 3	4	
R. Ga.	S	-	h	-	-	0 0 0	-	
	FP	-	h	-	-	0 0 0	-	
	RFP	h	±	-	-	0 0 0	-	
R. Co.	S	-	±	1a	4	4 4 4	4	
	SRCP	-	2, 3a	1a	4	4 4 4	4	
	RFP	4	±	3a	4a	4 4 4	4	
	RSP	2, 3a	1, 3a	1, 4a	4	4 4 2	2	
44R	SRCP	1, 2	4	1, 2	3	3 4 4	2	
	S	1	4	1, 2	4	4 4 4	2	

*Not tabulated are comparative serologic results obtained from the serums of spontaneously clotted blood specimens before and after saturation with secondary calcium phosphate. The latter showed no significant serologic titer alteration from those exhibited by SRCP. The question of the validity of the calcium concentration determinations in SRCP (because of the high inorganic phosphate content of these serums) is under study. At the present time they should be construed to have order of magnitude significance only.

†See footnote to Table I for key.

2. The serologic testing, other than that of the pilot serum specimens accompanying the blood units, was done at the Army Medical School, Division of Serology, where all specimens were subjected to a battery of tests made up of the Kline diagnostic and exclusion tests, the Mazzini, the Boerner-Jones-Lukens, and Kahn flocculation tests, and the Kolmer complement fixation test. Those specimens which were \pm plus on any of these tests were subjected to a quantitative procedure performed by successive dilutions of the serum or plasma; the determined reagin titer was expressed in units. Every attempt was made to secure approximate quantitative gradations in those specimens which showed less than a \pm plus degree of positivity. In this manner, good appraisal of serologic titer differences between plasma, serum, and recalcified plasma was obtained. These differences are shown in Table II and apparently do not offset the validity of the tests as performed on plasma or recalcified plasma.

RESULTS

In comparative serologic terms, results of tests performed on serum, citrated plasma, and recalcified citrated plasma, whether the latter was prepared by the addition of calcium chloride (Table I) or secondary calcium phosphate (Table II), indicate that from a practical standpoint all of these materials are suitable for serologic testing. In the few instances of undoubtedly false biologic reactions, these likewise were apparent in the citrated plasma and recalcified plasma, though it is noteworthy that their titer was not increased by calcification, as appeared to be the case in the true syphilitic specimens. The numbers of such instances are as yet too few to permit any general conclusions to be drawn of the significance of this finding or to justify its use as the basis of a verification procedure.

Determinations of calcium concentration made on recalcified citrated plasma showed that clotting may be induced without an inordinate rise in calcium content.

CONCLUSIONS

The collection of citrated blood samples for serologic testing for syphilis offers distinct advantages since citrated blood has better keeping qualities than whole clotted blood.

A procedure for the conversion of citrated whole blood or plasma to serum based on the specific clot-inducing properties of secondary calcium phosphate is described.

Serologic tests for syphilis conducted on citrated plasma or recalcified citrated plasma have the same validity as those done on serum.

REFERENCES

1. Laughlen, G. F.: Rapid Test for Syphilis, *Canad. M. A. J.* 33: 179-183, 1935.
2. Brown, A. L.: Personal communication.
3. Eagle, H. E.: The Laboratory Diagnosis of Syphilis, St. Louis, 1937, The C. V. Mosby Co., p. 74.
4. Rein, C. R., and Hazay, C. E.: Laughlen Test for Syphilis; Description of Certain Modifications, *J. Invest. Dermat.* 1: 283-293, 1938.
5. Ferguson, J. H.: A New Theory of Blood Coagulation, *Science* 97: 319-322, 1943.
6. Lubitz, J. M.: Serologic Reactions Following Smallpox Vaccinations, *Am. J. Clin. Path.* 13: 139-143, 1943.

THE APPLICATION OF A PROCESSED GLUE AS A SUBSTITUTE FOR GLASS COVERSLEIPS IN HISTOLOGIC TECHNIQUE*

MADUREIRA PARÁ, M.D.
RIO DE JANEIRO, BRAZIL

IN THE past several unsuccessful attempts have been made to discover an economical substitute for the glass coverslips used in mounting histologic specimens. Edinger,¹ in 1913, proposed the use of a glycerinated gelatin which, after application, was hardened with formol. This method, however, could not be employed in the presence of water-soluble aniline dyes, since the water used to dissolve the gelatin likewise dissolved the dyes. Lentze,² in 1930, and Bucholtz,³ in 1940, proposed the use of cellophane for covering specimens mounted in balsam. However, they both recognized that the method was impractical because of various technical difficulties. Suntzeff and Smith,⁴ in 1940, suggested the use of a special transparent plastic, a cellulose acetate called Plastacele, which is not affected by water or by fat solvents. Although the instructions for using this substitute were carefully followed, we observed, as was previously noted by others, that a considerable number of specimens were unsatisfactory for microscopic examination because of wrinkles at the edges and undulations in the center of the preparations. Likewise, in the course of time the specimens frequently became separated from the mountings and often became discolored after four or five months.

As a result of wartime conditions, glass coverslips have become scarce and expensive. Since Plastacele was found to be unsatisfactory, other materials were investigated, and one satisfactory substitute has been found. It consists of carpenter's glue subjected to a clearing and hardening process. The preparation and physical properties of the substance, which we have called "micromounting glue," as well as the method devised for its use, are described here.

PREPARATION OF MICROMOUNTING GLUE

Material.—(a) Commercial carpenter's glue, (b) potassium acetate (pure crystals), (c) egg white, (d) M/5 solution of potassium acid phosphate (prepared by dissolving 27.23 Gm. of KH_2PO_4 in 1,000 c.c. of double-distilled water).

Procedure.—(a) Thirty grams of carpenter's glue are broken into small fragments and placed in a 500 c.c. beaker together with a *fresh* solution of 4 Gm. of potassium acetate in 100 c.c. of distilled water. The beaker is then immersed in boiling water or in a glycerin bath at about 200°C . The latter method is the more rapid. It is allowed to remain until the glue is thoroughly dissolved.

Received for publication, Sept. 6, 1944.

*The work on which these observations are based was carried out under the auspices of the "Serviço de Estudos e Pesquisas sobre a Febre Amarela," which is maintained jointly by the Ministry of Education and Health of Brazil and the Rockefeller Foundation. The work was done in the Service of this

(b) The beaker is removed from the bath and the white of one egg is added. It is reimmersed for about five minutes and the mixture is stirred with a glass rod until the egg white is completely coagulated.

(c) The mixture is then filtered through three layers of gauze.

(d) A second filtration is made using coarse filter paper *

(e) Steps b, c, and d are repeated.

(f) The solution is allowed to cool and the glue is then neutralized with the M/5 KH_2PO_4 solution, using phenol red as an indicator.

(g) The glue is then placed in a wide-mouthed container of neutral glass (a beaker, for example) and kept in an incubator overnight at a temperature of 58°C ., which will reduce the volume to about one-half.

(h) The heavy yellowish white sediment which forms on standing overnight in the incubator should be removed by filtering through gauze or thin cloth.

(i) The glue is ready for use after filtering.

PHYSICAL PROPERTIES†

The glue has a high viscosity. It is limpid and similar to pure Canada balsam in color. It will keep for a long time in a well-stoppered dark vial in a cool place.

Micromounting glue has the following physical characteristics which are considered to be of importance in microscopie technique:

Color of solution (in Lovibond tintometer, 1-inch cell)	Combination of yellow 20 and red 10.4
Density at 25°C .	1.0903
Viscosity Engler $\frac{30^\circ}{20^\circ} \text{C}$.	5.0
pH at 25°C .	7.0
Refractive index of micromounting glue solution at 25°C . (Abbe Zeiss)	1.3840
Refractive index of dry micromounting glue at 25°C . (Abbe Zeiss)	1.4900
Mean transparency of dry micromounting glue on slide (using Pulfrich photometer and S 57 filter)	88%‡
Flexibility (180° angle)	No modification

Even after drying, the glue is easily soluble in water but is insoluble in alcohol, benzol, xylene, xylene-phenol, cedar oil, and other fat solvents.

TECHNIQUE OF APPLICATION

Apparatus for Dispensing Glue.—An apparatus has been devised for applying micromounting glue in liquid form to the sections which have previously been coated with Canada balsam and dried. Figs. 1 and 2 illustrate the construction and use of this apparatus. It consists of a metal cylinder (height, 8.5 cm.; internal diameter, 4.0 cm.) mounted on a base plate, at the upper end of which a glass container is inserted. This container serves as a reservoir for the glue. It has a hole in the center through which a metal shaft passes. This shaft has a square cup at the top (capacity, 1.6 c.c.; size,

*Arthur H. Thomas Co., No. 51601.

†The physical constants and properties of the micromounting glue have been determined through the kindness of personnel of the National Institute of Technology and the Division of Chemistry and Pharmacology of the Oswaldo Cruz Institute.

‡Under similar conditions, Canada balsam, with Arthur H. Thomas Co No. 1 glass coverslip, gave a mean reading of $D\% = 90$.

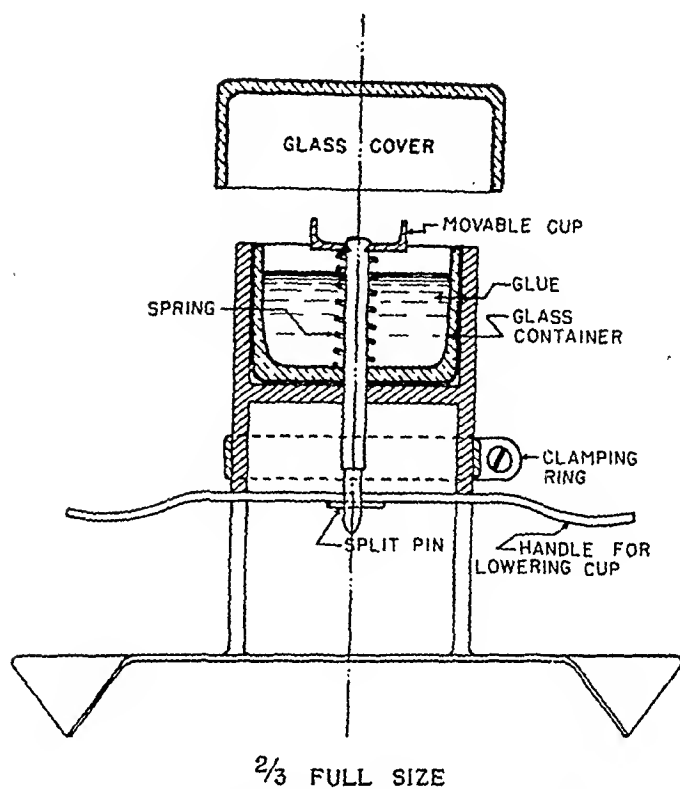


Fig. 1.—Working drawing of apparatus for applying micromounting glue in liquid form to sections.



Fig. 2.—Photograph of apparatus for applying micromounting glue, showing method of use.

18 × 18 × 5 mm.) which may be lowered for filling by means of a handle attached to the bottom of the shaft. When in position for applying glue, the cup is raised to a level slightly above the edge of the container by means of a spring mounted on the shaft between the square cup and the bottom of the glass container. The shaft can be secured in the filling position by means of a clamp so that the apparatus may be covered when not in use. All the metal parts are chromium plated to avoid rust.

Use of Glue With Hematoxylin-Eosin Stain.—The following process has been standardized in our laboratory for mounting sections stained with hematoxylin-eosin or a similar staining method.

1. Stained sections of paraffin-embedded tissue, affixed to slides, are dehydrated and placed in xylol as usual.

2. A slide is removed from the xylol and, after the latter has evaporated, one or two drops of a 50 per cent balsam-xylene mixture are applied by means of a thin glass rod, care being taken that the section of tissue is completely covered. Thus, a thin film of uniform thickness, with a transparent surface, is formed over the tissue section.

3. The slide is placed in an incubator in an exactly horizontal position with the section uppermost and allowed to remain for thirty minutes at a temperature of 56° C.

4. The liquid micromounting glue is then applied on top of the dry layer of balsam by means of the apparatus previously described. The movable cup is filled with glue and the slide, face downward, is brought obliquely in contact with the edge of the cup, turned down until it lies horizontally, and then removed and quickly inverted. The entire procedure should be one continuous and rapid movement. Care must be taken that the tissue section is well centered. It will be found that a uniform layer of glue, approximately 18 × 18 mm., has adhered to the slide. This may appear thicker than desirable, but subsequent drying reduces its volume to some extent, and also there is a slight spread.

5. The slide, again in a perfectly horizontal position, is allowed to dry in the incubator, face upward, for twenty minutes at 56° C.

6. The heat is then turned off, and fifteen minutes are allowed to pass with the incubator still closed.

7. The incubator is opened and, after fifteen to twenty minutes have elapsed and the temperature has fallen to 37 to 35° C., the slide may be taken out. The glue has now solidified, is thoroughly dry, and should be firmly adherent to the balsam and to the glass. The result is a smooth transparent film simulating a glass coverslip, with a thickness between 10 and 14 micra, and about 21 mm. square.

Use of Glue With Frozen Sections Stained With Sudan III.—For frozen sections, when stained with Sudan III or other similar dyes, the technique of mounting is as follows:

1. Stain the frozen section in the usual manner.
2. Remove the section from water and place it on a slide over which a thin layer of Meyer's albumin has been spread just previously.
3. Dry with absorbent paper.

4. Apply the micromounting glue in the manner described for use with paraffin sections but do not use Canada balsam.

5. Dry at 56° C. as previously described.

Preparations stained with Sudan III, mounted in this manner, are perfectly clear for microscopic examination and can be kept indefinitely.

GENERAL REMARKS

At first glance the application of micromounting glue would seem to be limited by the same disadvantages mentioned by Edinger regarding his gelatin mount: namely, that it could not be used in connection with specimens stained by water-soluble aniline dyes. This difficulty has been overcome, however, by an initial application of Canada balsam. The film is allowed to dry completely before the glue is applied. The glue adheres uniformly to the dry film of balsam, and it is not possible, even with the microscope, to detect any space between the two or to detect the presence of air bubbles.

For methods employing metallic impregnation, for hematoxylin stains, or for double-staining technique using simple dyes such as hematoxylin-eosin, van Gieson, Ziehl, the mounting process is carried out in an incubator regulated at a temperature of 56° C.

For complex staining methods or when thermolabile dyes such as Giemsa, Mann, Gram-Weigert, Masson's trichromic are used, the technique is the same, with one exception. It is then necessary to carry out the drying of the balsam and the glue at a temperature of 37° C., sixty minutes for the former and two hours for the latter. The double mounting in balsam and glue can, if necessary, be carried out at room temperature; however, in this case the balsam will require from one to two hours to dry completely, and the glue will require from two to three hours.

The method previously described may be employed to carry out the preparation, in series, of a considerable number of slides if special metal trays (for twenty-two slides each) are used for drying. With exception of the trays, the rest of the process for mounting a large number of slides is exactly similar to that described for mounting one slide.

Slides prepared by the process described have been filed in direct contact with one another within twenty or thirty days after mounting and have shown no alteration after more than one year.

Although, up to the present, only sections from small blocks of paraffin-embedded tissue have been mounted by this method, there seems to be no obvious reason why larger specimens should not be handled in a similar manner, regardless of the method of staining.

Tissue sections which have been mounted in the manner described can be examined with any degree of lens magnification, including the oil immersion lens.

This new method of mounting has also proved applicable to films for microbiologic or hematologic studies.

SUMMARY

A description is given of a process by which commercial carpenter's glue, clarified and hardened, may be used as a substitute for glass coverslips in histo-

logic technique. This glue is applied over a layer of Canada balsam, except for some special stains of frozen sections, which are mounted directly.

This substance has been termed "*micromounting glue*."

This method of mounting has its principal application in tissue sections, but it can also be used to preserve stained microbiologic smears.

Sections mounted in this manner have shown no alteration after a year. The method may be employed for the mounting of preparations for permanent record.

REFERENCES

1. Edinger, L.: Ersatz des Kanadabalsams durch Gelatine an mikroskopischen Präparaten, Neurol. Zentr. 32: 927, 1913.
2. Lentze, H.: Deckgläser für grosse histologische Schnitte (z.B. Hemisphärenschnitte) aus Cellophan, Klin. Wchnschr. 9: 1479, 1930.
3. Bucholtz, I.: Cellophane Cover Slips and a Method for Mounting, Science 92: 436, 1940.
4. Suntzeff, V., and Smith, I.: The Use of Plastic as a Substitute for Cover Glasses, Science 92: 17, 1940; 93: 157, 1941.

A NOMOGRAM FOR ORGAN WEIGHTS OF CHILDREN

HENRY W. EDMONDS, M.D.*
SEATTLE, WASH.

THE normal growth increment of organ weights in childhood is so appreciable that it would overshadow many variations due to disease if it were not specifically considered in the evaluation of weights observed at necropsy of a child. The figures for average weights of the major organs at various ages, supplied by Coppoletta and Wolbach,† provide standards to which observed weights can be compared. It has seemed desirable to mark directly upon the dial of the scales used at autopsy the organ weights considered normal for a given age. A graphic presentation of standards upon the scales dial makes possible the direct comparison of the observed and the expected weights, saving time for the pathologist and affording a visual demonstration for the clinician attending the autopsy. Such an age-weight chart, devised for the post-mortem room of the Children's Orthopedic Hospital, is to be presented here.

The scales employed is an ordinary commercial platter-type, fan-dial, springless balance manufactured for use on a market counter but eminently suitable for the post-mortem room. The fan-shaped dial is calibrated in pounds and ounces, with a numerical nomogram giving dollar-and-cents values. A moving bar indicator, passing radially over the dial, is marked so that from it the dollar-and-cents value of the commodity on the scales platter can be read at various price levels (45 cents a pound, for example) designated on the bar. Since one pound avoirdupois is the equivalent of 0.4536 kilogram metrie, it is obvious that the dial furnished on the scales will give direct metrie readings in the segment of the nomogram opposite the 45-cents-a-pound mark on the moving bar, if one neglects an error of 3.6 Gm. per kilogram. Actually this inaccuracy can be corrected by a slight adjustment of the scales machinery by a scales mechanic.

The special nomogram of normal organ weights is illustrated in Fig. 1. It is designed to be pasted over the dollar-and-cents nomogram on the scales dial, as shown in Fig. 2. In the special chart a gap is left as a window, through which the original markings on the 45-cents-a-pound line can be read. This section of the dial gives the actual metric weight of the organ being examined—metric weight at forty-five deci-kilograms per pound. On the remainder of the special nomogram normal organ weights were plotted graphically against the various ages. The weights were entered on radii increasing from left to right, while the ages were entered on concentric circular segments increasing from top to bottom. The actual lines of the finished nomogram are smoothed curves, based on the plotted points of the Coppoletta-Wolbach weights. Another paster

Received for publication, Aug. 12, 1944.

*Pathologist, the Children's Orthopedic Hospital.

†Coppoletta, Joseph M., and Wolbach, S. B.: Body Length and Organ Weights of Infants and Children, *Am. J. Path.* 9: 55, 1933.

strip, also shown in Fig. 1, designed to be affixed over the indicator bar of the scales, carries numbers corresponding to the various ages of the range included, increasing in order from top to bottom

When a brain, for example, is placed on the scales platter, the moving indicator bar moves automatically so that it lies adjacent to the value of the actual metric weight of the brain in the window segment of the nomogram and adjacent to the value of the average weight of a brain for the specific age con-

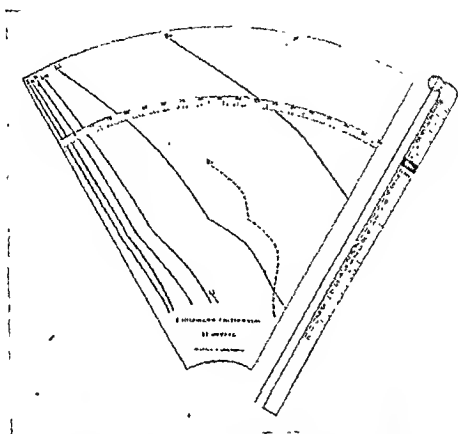


Fig. 1.—A nomogram giving average weights of the major organs in the age range from birth to 12 years. The fan chart is pasted on the dial of the scales, with the metric weights (in deci-kilograms) indicated in the narrow circular segment. The strip bearing age values in vertical order (birth, 1 through 16 weeks, 6 through 24 months, and 3 through 12 years) is pasted on the indicator bar of the scales. *S.*, Spleen; *H.*, heart; *K.*, kidneys; *Lu.*, lungs; *Li.*, liver; *Br.*, brain

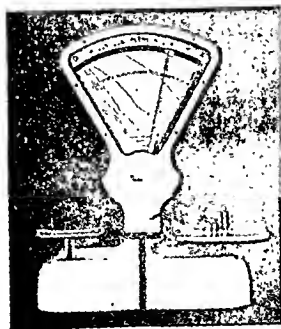


Fig. 2.—The nomogram dial and indicator bar affixed to a commercial platter-type, fan-dial, springless balance for a direct comparison of observed organ weights and weights considered average for the organs at the specific age, as well as for weighing in grams.

sidered in the segment of the nomogram opposite the specific age as marked on the bar. If the brain is of average weight for the age, then the bar margin will coincide with the line for brain weights (line *Br* on the nomogram). If the brain is above or below average weight, the bar margin will be found to the right or to the left of the *Br* line, respectively, in the zone corresponding to the age of the child examined. The proportional extent of overweight or underweight is evident at a glance.

Since the indicator bar moves when the scales platter is displaced (either by the weight of an organ or by pressure of a finger), the bar can readily be moved over so that the part of it marked for a particular age adjoins the line for a given organ. In this position the part of the indicator bar over the window segment will adjoin the corresponding metric weight, allowing a direct reading of the average weight of the selected organ at the selected age.

It will be noted that the line on the nomogram representing brain (*Br*) is discontinuous. There is an apparent setback in weight of this organ between the ages of 12 and 14 months. This is an artefact explained as follows: The scales dial reads up to two pounds. Larger weights can be read if a tare weight is added to an auxiliary platter (other scales of somewhat different design accomplish this by a tare weight sliding on a beam). For weighing a brain of a child older than 12 months (or any other organ weighing more than two pounds) a 500 Gm. tare weight is added to the auxiliary platter to bring the indicator bar back to the middle of the dial where the nomogram line for older (heavier) brains is placed. A similar maneuver is necessary in the case of the liver of a child older than 12 years; note corresponding break in the liver line (*Li* on the nomogram).

The nomogram values for the paired organs, lungs and kidneys, are those for both right and left lungs (or kidneys) weighed together.

The nomogram described has proved serviceable at the Children's Orthopedic Hospital. It has been presented in the hope that others may find it of similar help and that it may increase the usefulness of the Coppoletta-Wolbach average weight values.

CHEMICAL

A SIMPLE METHOD FOR ESTIMATING SERUM ATABRINE CONCENTRATION

CAPTAIN ROGER A. LEWIS
MEDICAL CORPS, ARMY OF THE UNITED STATES

A METHOD has been devised for the rapid estimation of serum atabrine level employing the equipment available in an overseas general hospital. With this technique, over ten thousand determinations have been performed during the past year. The equipment and reagents when crated for air shipment weigh less than 200 pounds. A source of electricity and water is required. The chief reagents are petroleum ether and chloral hydrate. The major items of equipment are a small centrifuge and a portable ultraviolet lamp. It has been possible to set up the equipment in a combat area in twenty-four hours and to operate in the heat and humidity of an island within a few degrees of the equator.

The fluorescence of atabrine may be detected in very dilute solutions, particularly in a solvent such as chloral hydrate. However, normal serum contains other fluorescent substances which must be removed. The separation of atabrine from other substances is based upon its solubility in acid aqueous solution and its relatively increased solubility in petroleum ether when the aqueous phase is rendered alkaline. When serum is shaken with petroleum ether, a gelatinous layer forms which may be broken up by adding a few drops of absolute ethyl alcohol.

Unknown sera are compared with standard solutions of atabrine in water that have been similarly extracted. The comparison is made in ultraviolet light in a dark room. The light must be equipped with a Wood's glass filter to prevent the transmission of visible light. Readings are easy to perform if the final solutions are compared in a wooden rack with an oblique base that has been painted black.

PROCEDURE

Fifteen cubic centimeters of blood are withdrawn from the fasting patient and placed in a 15 c.c. centrifuge tube. After the blood has clotted, it is ringed with a glass rod and centrifuged for fifteen minutes. The serum is decanted into a Wasserman tube and recentrifuged for five minutes. It is then placed in a 15 c.c. centrifuge tube that has been calibrated at 5 and 11 c.c. so that any excess serum may be pipetted off.

In similar tubes, .1, .2, .3, .5, 1.0, and 2.0 c.c. of a .05 mg. per 100 c.c. solution of atabrine are placed to correspond to a final dilution of .001, .002, .003, .005,

Received for publication, Sept. 28, 1944.

.010, and .020 mg. per 100 c.c. The .05 mg. per 100 c.c. solution is made up fresh each day by a fifty-fold dilution of the stock standard. The latter is made up from atabrine dihydrochloride to a concentration of 2.5 mg. per 100 c.c. and kept in a dark bottle in a cool place. It should be noted that the primary standard is atabrine dihydrochloride rather than the free base.

To the unknown and standard solutions 1 c.c. of 1 normal sodium hydroxide and 5 c.c. of petroleum ether are added. The latter should be redistilled, the fraction boiling below 50 or over 70° C. being rejected. The tubes are corked with specially prepared rubber stoppers and shaken five times, first gently, then vigorously. The alkali and petroleum ether are added to six tubes which are then shaken and centrifuged for five minutes while the next six tubes

TABLE I

RESULTS

DATE	MEDIUM	THEORETICAL (MG. PER 100 C.C.)	RECOVERY (MG. PER 100 C.C.)	% ERROR
8/ 9	Plasma	.025	.027	8
8/ 9	Plasma	.000	.000	0
8/17	Plasma	.020	.020	0
8/17	Plasma	.004	.004	0
8/25	Plasma	.030	.035	17
8/31	Serum	.010	.011	10
9/ 4	Plasma	.003	.003	0
9/ 7	Plasma	.003	.003	0
9/ 7	Plasma	.004	.004	0
9/ 7	Plasma	.015	.015	0
9/ 8	Plasma	.030	.027	-10
9/ 9	Plasma	.012	.009	-25
9/ 9	Plasma	.018	.015	-17
9/10	Serum	.010	.012	20
9/10	Plasma	.005	.004	20
9/11	Plasma	.005	.004	20
9/12	Plasma	.003	.003	0
9/13	Plasma	.017	.018	6
9/14	Plasma	.002	.002	0
9/15	Plasma	.009	.009	0
9/16	Plasma	.016	.015	-6
9/18	Plasma	.007	.007	0
9/19	Plasma	.004	.006	50
9/20	Plasma	.015	.017	12
9/21	Plasma	.012	.012	0
9/22	Plasma	.020	.020	0
9/23	Plasma	.008	.008	0
9/24	Plasma	.018	.018	0
9/25	Plasma	.004	.004	0
9/26	Plasma	.003	.002	50
9/27	Plasma	.017	.020	18
9/28	Plasma	.030	.030	0
9/29	Plasma	.012	.012	0
9/30	Plasma	.020	.020	0
10/ 1	Plasma	.005	.005	0
10/ 2	Plasma	.010	.009	-10
10/ 4	Plasma	.010	.011	10
10/ 4	Plasma	.020	.019	-5
10/ 6	Plasma	.030	.035	17
10/ 7	Blood	.020	.020	0
10/ 7	Blood	.020	.021	5
10/ 8	Plasma	.020	.020	0
10/ 8	Blood	.020	.020	0
10/ 9	Plasma	.010	.009	-10
10/10	Plasma	.020	.018	-10
10/11	Plasma	.010	.009	-10
Total number	45		Average error	8%

are being prepared and shaken. A few drops of absolute ethyl alcohol are added to the layer which forms, and the tubes are again centrifuged for five minutes. It is helpful to agitate the gelatinous layer with a glass rod after adding the alcohol.

White stoppers* were used to cork the serum petroleum ether mixture. It is necessary to soak them for fifteen minutes in petroleum ether and to boil them for fifteen minutes in a very dilute alkali several times. To reduce further chances of contamination from traces of wax pencil, the tubes were numbered and graduated with a diamond pencil.

A 4 c.c. aliquot from each of the petroleum ether extracts is placed in a clean dry centrifuge calibrated at 4 c.c. for that purpose. Then 1 c.c. of a saturated aqueous solution of chloral hydrate is added to each tube. Each tube is corked with a rubber stopper that has been wiped with chloral hydrate and shaken briskly for one minute. One cork is sufficient for all the tubes if it is wiped carefully. The tubes are then centrifuged for five minutes and the petroleum ether suctioned off. The tubes are again centrifuged for five minutes to clear the extract which is then ready for reading.

In comparing the unknown extracts with the extracts of the standard solutions, it is advisable to stand directly behind the ultraviolet light. The latter should be switched on five minutes prior to use. A violet fluorescence indicates the presence of quinine. A blue-gray fluorescence shows that there has been contamination. If neither of these is present it is comparatively easy to match the green fluorescence of atabrine against the standard extracts although they both contain a slight gray fluorescence due to dissolved gases. Occasional values may be interpolated, although for clinical purposes readings under .001 mg. per 100 c.c. are referred to as a trace.

When the standards are made up with distilled water it is advisable to run control solutions made up in serum. These control studies form the bases of Table I.

DISCUSSION

This method, though relatively simple, requires considerable care. Inasmuch as the cellular constituents of blood have a high atabrine content, it is essential that the serum or plasma be entirely free of these elements. Agitation of the serum and petroleum ether must be very thorough. Efficient extraction is marked by a transient blue color which develops when the petroleum ether and chloral hydrate are shaken. This will not occur if the chloral hydrate is not saturated. The cleanliness of the glassware and rubber stoppers is very important. The latter may be cleaned in petroleum ether and allowed to stand in dilute alkali. Chloral hydrate, sodium hydroxide, and alcohol do not usually require any purification. The petroleum ether and the water must be redistilled. When massive treatment is given, higher standards are needed, and when suppressive levels are measured, lower standards afford an advantage. The preparation of serum or plasma is as time consuming as the actual extraction. The final reading takes about fifteen minutes. Two persons can perform fifty determinations daily, collecting the specimens and cleaning the glassware.

*Obtained from Sharp & Dohme plasma sets.

.010, and .020 mg. per 100 c.c. The .05 mg. per 100 each day by a fifty-fold dilution of the stock standard from atabrine dihydrochloride to a concentration kept in a dark bottle in a cool place. It should be used as atabrine dihydrochloride rather than the free base.

To the unknown and standard solutions 1 c.c. of iodine and 5 c.c. of petroleum ether are added. The mixture is allowed to boil below 50 or over 70° C. and is then corked with specially prepared rubber stopper and shaken gently, then vigorously. The alkali and petroleum ether are then shaken and centrifuged for five minutes.

TABLE I

RESULTS

DATE	MEDIUM	THEORETICAL (MG. PER 100 C.C.)
8/ 9	Plasma	.025
8/ 9	Plasma	.000
8/17	Plasma	.020
8/17	Plasma	.004
8/25	Plasma	.030
8/31	Serum	.010
9/ 4	Plasma	.003
9/ 7	Plasma	.003
9/ 7	Plasma	.004
9/ 7	Plasma	.015
9/ 8	Plasma	.030
9/ 9	Plasma	.012
9/ 9	Plasma	.018
9/10	Serum	.010
9/10	Plasma	.005
9/11	Plasma	.005
9/12	Plasma	.003
9/13	Plasma	.017
9/14	Plasma	.002
9/15	Plasma	.009
9/16	Plasma	.016
9/18	Plasma	.007
9/19	Plasma	.004
9/20	Plasma	.015
9/21	Plasma	.012
9/22	Plasma	.020
9/23	Plasma	.008
9/24	Plasma	.018
9/25	Plasma	.004
9/26	Plasma	.003
9/27	Plasma	.017
9/28	Plasma	.030
9/29	Plasma	.012
9/30	Plasma	.020
10/ 1	Plasma	.005
10/ 2	Plasma	.010
10/ 4	Plasma	.010
10/ 4	Plasma	.020
10/ 6	Plasma	.030
10/ 7	Blood	.020
10/ 8	Plasma	.020
10/ 8	Blood	.020
10/ 9	Plasma	.010
10/10	Plasma	.020
10/11	Plasma	.010
Total number		45

AUTHORS INDEX

△

- II

- JORDAN, ARTHUR

- C

- 1309

- CHASE, AURIN M., LORENZ, PHILIP B., PART, ARTHUR K., AND GREGG, JOHN R. A method for simultaneous determination of methemoglobin-oxyhemoglobin ratio and per cent hemolysis in stored blood, 99
- CHASE, H. F. (See LEHMAN AND CHASE), 561
- CHRISTENSEN, W. BLAKE. A medium for determination of citrate utilization by colon bacteria, 366
- CLARK, BYRON B. (See VAN LOON AND CLARK), 942
- CLARKE, CARL DAME. Facial and body prosthesis in relation to war wounds, 667
- (See CONVERSE, CLARKE, AND GUIDI), 546
- COCA, ARTHUR F. The adjuvant effect of aerosol upon the germicidal action of cadmium chloride, 689
- COHN, ESSIE WHITE, AND ROE, JOSEPH H. A new technique for the measurement of glycogenesis: a study of glycogen metabolism in the liver under fasting conditions, 196
- COLE, VERSA V. (See HULPIEU AND COLE), 1134
- COLEMAN, HAROLD M. (See McNALLY AND COLEMAN), 429
- COLEMAN, MARION B. Vi agglutinative properties for *Bacterium typhosum* demonstrated following infection with malaria parasites, 916
- COLLIER, H. BRUCE, AND WILBUR, KARL M. Lysolecithin and the antihemolytic value of the blood, 1123
- CONVERSE, JOHN MARQUIS, CLARKE, CARL DAME, AND GUIDI, HENRI. Repair of cranial defects by cast chip-bone grafts, 546
- COPE, H. E. (See GOTTSCHALL, DEKRUIF, COPE AND LAURENT), 614
- COPLEY, ALFRED L., AND WHITNEY, DAVID V., III. The standardization and assay of heparin by the toluidine blue and azure A reactions. (A correction), 117
- CORPER, H. J. Comparative results with transdermal (or transcutaneous) and intracutaneous tuberculin tests, 398
- Co Tui, HOPE, D., SCHRIFT, M. H., AND POWERS, J. Purified pyrogen from *Eberthella typhosa*, 58
- CRAFTS, ROGER C. Effects of ether anesthesia upon total erythrocyte and white cell counts of adult female rats, 1070
- CRITTENDEN, PHOEBE J., AND JOINER, LUELLA S. Cotton hose as a vehicle for a fungicide in treatment of athlete's foot, 606
- D
- DAMON, SAMUEL R., AND JOHNSON, MARY B. A rapid agglutination test for the diagnosis of tularemia, 976
- DE ANGELIS, EUGENE, AND HUNTSINGER, MILBRED. The clumping of erythrocytes in Hayem's diluting fluid: a grave prognostic sign, 624
- DEKRUIF, P. (See GOTTSCHALL, DEKRUIF, COPE, AND LAURENT), 614
- DENHOFF, ERIC, AND PIPER, BERNARD C. Laboratory aids in the diagnosis of malaria, 518
- DENKO, CHARLES W., AND ANDERSON, ARTHUR K. Studies on the toxicity of gold compounds in rats, 1168
- DE NOSAQUO, NORMAN, KERLAN, IRVIN, KNUDSEN, LILA F., AND KLEMPPE, THEODORE G. Clinical use of oral thermometers, 179
- DERBES, VINCENT J., AND ENGELHARDT, HUGO T. Deaths following the use of local anesthetics in transcrioid therapy: a critical evaluation, 478
- DERNEHL, CARL U., BADER, MADERO N., AND BARTLETT, LUIS H. A simple inexpensive apparatus for the sterile lyophilization of small amounts of material, 978
- DIETZ, ALBERT. (See STEINBERG AND DIETZ), 695
- DI PALMA, JOSEPH R., AND KENDALL, PHILIP E. The relationship between blood volume and blood specific gravity in the recovery from cardiac decompensation, 390
- DOAN, CHARLES A. (See SNYDER AND DOAN), 1211
- DREISBACH, ROBERT H. (See PFEIFFER, DREISBACH, AND ROBY), 709
- DRURY, WALTER. (See TOOMEY, LEWIS, AVERILL, DRURY, AND TAKACS), 21
- DUNHAM, WOLCOTT B. (See PARKER, DUNHAM, AND MACNEAL), 37
- E
- EDMONDS, HENRY W. A nomogram for organ weights of children, 1300
- ELIASER, MAURICE, JR. Vertigo and related conditions, 680
- ELLIOTT, K. A. C. (See RIVERS AND ELLIOTT), 55
- ELMAN, ROBERT. (See ROBERTS, HELWIG, ELMAN, AND MACBRYOE), 1062
- ELVERS, CHARLES F. Polychrome No. 28. A simplified polychrome stain for use in the clinical laboratory, 1094
- ENGELFRIED, JOHN J. The ascorbic acid saturation test, 324
- ENGELHARDT, HUGO T. (See DERBES AND ENGELHARDT), 478
- ENZER, NORBERT, SIMONSON, ERNST, AND BLANKSTEIN, SAMUEL S. The reappearance of flicker at high flash frequency in patients with brain pathology and in normal subjects, 63
- EPSTEIN, JEANNE A., FOLEY, E. J., PERRINE, ISABEL, AND LEE, S. W. Convenient assay method for penicillin and similar substances, 319
- F
- FALKENSTEIN, ALFONS P. (See RAMSEY, FALKENSTEIN, AND SUJKOWSKI), 419
- FAVORITE, GRANT O., REINER, LEOPOLD, AND LONDON, RUSSELL. Acute agranulocytosis during sulfamerazine therapy, 899
- FEINBLATT, HENRY M., ALPERT, BARNETT J., AND FERGUSON, EDGAR A., JR. Juvenile diabetes insulin sensitivity, 366
- FELLOVES, OLIVER N., AND ROUTH, JOSEPH I. A study of the chemical composition and anti-*poly-saccharid* cultures of 1054
- FELSFELD, OSCAR, AND YOUNG, VIOLA MAE. The occurrence of members of the genus *Salmonella* in inhabitants of state hospitals of the greater Chicago area, 375
- FERDERBER, MURRAY B. (See MOSES AND FERDERBER), 1147
- FERGUSON, EDGAR A., JR. (See FEINBLATT, ALPERT, AND FERGUSON), 366
- FIGGE, FRANK H. J. *Unpublished* THOMAS E., JR. publishing phenomena
- FINCH, EDMOND P., JR. (See SCOTT AND FINCH), 404
- FISHBACK, HAMILTON R. On the mobilization of iron in hemochromatosis with administration of various chemicals, 742
- FISHER, C. VIRGINIA. (See MARTIN AND FISHER), 383
- FITTS, LUCILLE. (See TUTTLE AND FITTS), 609
- FLANAGAN, MARY E. (See LOUGHLIN, BENNETT, AND FLANAGAN), 568
- (See LOUGHLIN, BENNETT, FLANAGAN, AND SPITZ), 921

- FLORIN, R. E. AND SILVERSTEIN, R. M. Sulfonamide determination in blood using nylenesulfonic acid as a protein precipitant, 879
- FOLLEY, E. J. (See EPSTEIN, FOLLEY, FERRINE, AND LER), 319
- FOLLEY, JOHN A. (See KROPP AND FOLLEY), 231
- FREE, ALFRED H., AND LEONARDS, JACK R. Studies on the ingestion of large quantities of protein and amino acids, 963
- FRIEDMAN, M. H. F. A vehicle for the intravenous administration of fat-soluble hormones, 330

G

- GATCH, W. D. (See WAKIM AND GATCH), 984
- GELARIE, ARNOLD J. Cultivation and preservation of diplococcus pneumoniae and mycobacterium tuberculosis in market eggs, 532
- GILL, A. J. Local eosinophilia in malignant neoplasms, 820
- , Traumatic autograft of splenic tissue in the body wall, 247
- GOETCHIUS, G. R. AND LAWRENCE, C. A. The antibacterial effects of several acidine compounds, 1177
- AND —, The antibacterial effects of various acidine compounds, 1341
- GOLDFARB, A. I. (See SHAPIRO, TARLOV, OLIVER, GOLDFARB, BOJAR, KASLOW, AND ROCKENMACHER), 282
- GOTTSCHALL, R. Y., DEKRUIF P., COPE, H. E., AND LAURENT, D. The effect of artificially induced fever on anaphylactic shock in actively sensitized guinea pigs, 614
- GRAU, MANOUERITE L. A simple acid water solution for better visualization of malarial parasites using Wright's stain for thick blood films, 1103
- GREGORSEN, MAGNUS I. A practical method for the determination of blood volume with the dye T-1834, 1266
- GREGO, JOHN R. (See CHASE, LORENZ, PARFANT, AND GREGO), 99
- GREIFINGER, WILLIAM, AND SILVERSTEIN, JOSEPH K. Salmonella food infection in military personnel, 1042
- GUIDI, HENRI. (See CONVERSE, CLARKE, AND GUIDI), 546
- VON GUFFELD, F. J. Method to determine the penetrating power of germicides, 413

H

- HALL, BETTY L. Botulism from home-canned beets, 702
- HAMBLETON, A. A time and temperature guide for inspissating culture media, 526
- HARRIS, ALBERT H. (See LANGE AND HARRIS), 970
- HARRIS, ALBERT M., AND PRIESTLEY, JOSEPH B. Actinomycosis, 815
- HART, FANCHON, AND BLUMBERG, BERNARD L. A simple apparatus for periodic testing of *P. notatum* flask cultures, 749
- HEINEMANN, BERNARD. The bactericidal activity of some di-(hydroxyphenyl) alkanes, 254
- HELWIG, ELSON B. (See ROBERTS, HELWIG, ELMAN, AND MACBRIDE), 1062
- HELWIG, FERDINAND C. A simple rapid method for paraffin tissue preparations utilizing the Kahn shaker, 304
- HELZ, MARY K., AND MENTEN, MAUDE L. Erythrocytosis, a report of two cases, 185
- HENINGWAY, ALLAN, AND TAYLOR, CHARLES B. Laboratory tests for the oximeter with automatic compensation for vasomotor changes, 987
- HENNELMANN, LOUIS A., JR., REINHARD, EDWARD H., MOORE, CARL V., BIERDAUS, OLGA S., AND MOORE, SHERWOOD. Hematologic complications of therapy with radioactive phosphorus, 1020
- HERRMANN, GEORGE R. (See RUSKIN AND HERRMANN), 486
- HESS, MANFRED, AND HOLLANDER, FRANKLIN. Permanent metachromatic staining of gastric mucus smears, 321
- HILL, J. M. (See MUIRHEAD AND HILL), 339

- HINSCHBERG, NELL. A solid medium for the transportation of delayed gonococcus cultures, 311
- HORTMARTER, THOMAS E., JR. (See FIGGE AND HOFFMARTER), 1207
- HOLLANDER, FRANKLIN. (See HESS AND HOLLANDER), 321
- HOLMES, LIDA F., AND WILSON, MARY E. Gentian violet-blood agar plates used in aerobic and anaerobic cultures of wounds, 1090
- HOPE, D. (See CO TRI, HOPE, SCHRIFF, AND POWERS), 58
- HORN, J. MORRIS. A note on a simplified and rapid test for proteinuria, 228
- HUEPEN, W. C. Reactions in the blood and organs of dogs on intravenous injection of a solution of hemoglobin, 628
- HUPLIET, H. R., AND COLE, VERSA V. The effect of humidity and temperature on oxygen toxicity, 1134
- HUNT, GEORGE A. A study of the Pappenheim stain—a stable modification, 207
- HUNTER, ALBERT C. (See WELCH, PRICE, NIELSEN, AND HUNTER), 809
- HUNTSINGER, MILDRED. (See DE ANGELIS AND HUNTSINGER), 624

I

- IYI, A. C. (See ANNENBERG, SNAPP, IYI, AND ATKINSON), 858

J

- JACKSON, D. E. The pharmacologic action of 2-methylaminoheptane (EA-1), 150
- JACOBS, JOHN, AND YONKMAN, FREDRICK F. Sympatholytic treatment of experimental hypertension, 1217
- JAFFE, RUDOLF. General considerations on pathogenesis, syphilitic aortitis, myocarditis, hepatic cirrhosis, 139
- JANDORF, BERNARD J. (See WILLIAMS, JANDORF, AND KAY), 329
- JAWETZ, E. A mechanical iodine applicator, 664
- JOHNSON, HAROLD N. Isolation of *Bacterium tularensis* from the sputum of an atypical case of human tularemia, 903
- , Natural occurrence of tularemia in dogs used as a source of canine distemper virus, 906
- JOHNSON, MARY B. (See DAMON AND JOHNSON), 976
- JOHNSON, MARY S. (See SHULMAN AND JOHNSON), 500
- JOINER, LUELLA S. (See CRITTENDEN AND JOINER), 606
- JORDAN, ANTHONY C. (See BERNHARD AND JORDAN), 273, 357

K

- KALMANSON, G. M., AND BRONFENBRENNER, J. J. Reversibility of sensitization of erythrocytes, 684
- KASLOW, R. (See SHAPIRO, TARLOV, OLIVER, GOLDFARB, BOJAR, KASLOW, AND ROCKENMACHER), 282
- KAY, GLORIA A. (See WILLIAMS, JANDORF, AND KAY), 329
- KENDALL, PHILIP E. (See DI PALMA AND KENDALL), 390
- KENDALL, H. W. (See GOTTSCHALL, DEKRUIF, COPE, AND LAURENT), 614
- KERLAN, IRVIN. (See DE NOSAQUO, KERLAN, KNUDSEN, AND KLUMPF), 179
- KINGSLEY, GEORGE R. The adaptation of the direct buret method for the determination of serum proteins to box comparator colorimetry, 436
- , The preparation of a more stable concentrated urease solution, 337
- KINSEY, ROY E., AND WRIGHT, EDWARD O. Reaction following ingestion of sodium benzoate in a patient with severe liver damage, 188
- KLUMPF, THEODORE G. (See DE NOSAQUO, KERLAN, KNUDSEN, AND KLUMPF), 179
- KNUDSEN, LILA F. (See DE NOSAQUO, KERLAN, KNUDSEN, AND KLUMPF), 179
- KNOSS, MELVIN E. The laboratory diagnosis of tuberculosis, 75

- KORNBERG, HARRY A. A simple automatic pipette, 747
- KRAUEL, KATHRYN K. The microdetermination of amino acid nitrogen in blood with the spectrophotometer and with the optical colorimeter, 222
- KRAUTMAN, BENJAMIN. (See SIFFERT AND KRAUTMAN), 270
- KROPP, BENJAMIN. (See REED AND KROPP), 214
- KROPP, GEORGE V., AND FOLEY, JOHN A. Studies on a polysaccharide from the tubercle bacillus, 231
- L
- LANGE, CARL, AND HARRIS, ALBERT H. The significance of the pH in the gold reaction, 970
- LAUBER, FRANCES U., AND MATTICE, MARJORIE R. Microdetermination of blood glucose, 113
- LAUG, EDWIN P. A multiple rabbit holder, 308
- LAURENT, D. (See GOTTSCHALL, DEKRUIF, COPE, AND LAURENT), 614
- LAWRENCE, C. A. (See GOETCHUIS AND LAWRENCE), 134, 1177
- LAYNE, JOHN A., AND SCHEMM, F. R. Acute macrocytic hemolytic anemia occurring following administration of sulfadiazine, 347
- LEADER, S. A. Coronary disease associated with a short PR interval and a prolonged QRS, 673
- LEE, H. I. Comparison of procedures for staining tubercle bacilli in fluorescence microscopy, 218
- LEE, S. W. (See BRSTEIN, FOLEY, PERRINE, AND LEE), 319
- LEHMAN, A. J., AND CHASE, H. F. The acute and chronic toxicity of isopropyl alcohol, 561
- LEIGHTY, JOHN A. (See WICK AND LEIGHTY), 303
- LENHOFF, CHARLES D. (See SCHNITKER AND LENHOFF), 889
- LEONARDS, JACK R. (See FREE AND LEONARDS), 963
- LEWIS, NORMAN. (See TOOMEY, LEWIS, AVERILL, DRURY, AND TAKACS), 21
- LEWIS, ROGER A. A simple method for estimating serum atabrine concentration, 1303
- LILLIE, R. D. Factors influencing the Romanovsky staining of blood films and the role of methylene violet, 1181
- LINTON, LORRAINE GROSCHIN. (See CALLOMON, FRITZ T.), 574
- LITTLE, J. MAXWELL. A simple ventilation recorder for animal experiments, 1205
- LONDON, RUSSELL. (See FAVORITE, REINER, AND LONDON), 899
- LORENZ, PHILIP B. (See CHASE, LORENZ, PARPALT, AND GREGG), 99
- LOUGHLIN, ELMER H., BENNETT, RICHARD H., AND FLANAGAN, MARY E. The treatment of pneumococcal pneumonia with sulfamethazine, 568
- , —, AND SPITZ, SAMUEL H. Studies on the absorption of the sulfonamides from the gastrointestinal tract of albino rats, 921
- LUISADA, ALDO A. (See RAPPAPORT AND LUISADA), 638
- LUSK, FRANK B. (See BOHAN AND LISK), 385
- M
- MCBRIDE, ALICE. (See ROWE, MCBRIDE, AND METTIER), 1156
- MACBRYDE, CYRIL M. (See ROBERTS, HELWIG, ELMAN, AND MACBRYDE), 1062
- MCGAVACK, THOMAS HODGE, AND VOGEL, MILDRED. Propylene glycol as a menstruum for the administration of steroid hormones, 1256
- MCKIBBIN, J. M., THAYER, S., AND STARE, F. J. Choline deficiency studies in dogs, 1109
- MENALLY, WILLIAM D., AND COLEMAN, HAROLD M. A micromethod for the determination of ethyl alcohol in blood, 429
- MACNEAL, WARD J. (See PARKER AND MACNEAL), 121
- (See PARKER, DUNHAM, AND MACNEAL), 37
- MCWILLIAMS, MARY A. (See ENGELFRIED, JOHN J.), 324
- MADONICK, M. J., AND SAVITSKY, NATHAN. The relation between the Pandey test and total protein content of spinal fluid, 542
- MALLERY, O. T., JR. (See RANDOLPH AND MALLERY), 197
- , AND RANDOLPH, THERON G. The effect in vitro of propylene glycol in leucocytes, 203
- MARTIN, GUSTAV J., AND FISHER, C. VIRGINIA. Nutritional status of *Staphylococcus aureus* as influenced by proflavine, 383
- MATTICE, MARJORIE R. (See LAUBER AND MATTICE), 113
- (See PINCK, MORTON, AND MATTICE), 462
- MENTEN, MAUD L. (See HELZ AND MENTEN), 185
- MERANZE, DAVID R. (See TRASOFF AND MERANZE), 590
- MERRILL, MALCOLM H. (See NEUFELD AND MERRILL), 1100
- METTIER, STACY R. (See ROWE, MCBRIDE, AND METTIER), 1156
- MINNICH, V. (See MOORE, VILTER, MINNICH, AND SPIES), 1226
- MINOT, ANN S. The determination of tocopherol in blood serum, 772
- MOORE, CARL V. (See HEMPELMANN, REINHARD, MOORE, BIERBAUM, AND MOORE), 1020
- , VILTER, R., MINNICH, V., AND SPIES, T. D. Nutritional macrocytic anemia in patients with pellagra or deficiency of the vitamin B complex, 1226
- MOORE, SHERWOOD. (See HEMPELMANN, REINHARD, MOORE, BIERBAUM, AND MOORE), 1020
- MORTON, GLADYS L. (See PINCK, MORTON, AND MATTICE), 462
- MOSES, CAMPBELL, AND FERDERBER, MURRAY D. The oscilometer and thermocouple as diagnostic aids in peripheral vascular disease, 1147
- MUIRHEAD, E. E., AND HILL, J. M. Shock resulting from the intraperitoneal implantation of reconstituted desiccated muscle, 339
- MUNRO, F. L. A modified pipette for prothrombin determinations, 986
- MYERS, VICTOR C. The prognostic significance of an elevated blood creatinine, 1001
- N
- NECHELES, H., AND OLSON, W. H. Note on the "digestion" of metal in the stomach, 687
- NELSON, TRUMAN. (See RAY AND NELSON), 766
- NEUFELD, JOHN W., AND MERRILL, MALCOLM H. A mechanical device for making multiple paraffin rings for slide precipitation tests for syphilis, 1100
- NIELSEN, JEAN K. (See VEECH, PRICE, NIELSEN, AND HUNTER), 809
- O
- O'BRIEN, ELWIN J. (See BROWN, O'BRIEN, AND ROOK), 410
- OLIVERIA LIMA, A. Allergy from timbó (*Lonchocarpus H.B.K.*), 929
- OLIVER, R. (See SHAPIRO, TARLOV, OLIVER, GOLDFARB, BOJAR, KASLOW, AND ROCKENMACHER), 282
- OLSON, W. H. (See NECHELES AND OLSON), 687
- ORR, MARTHA L. (See TORIE AND ORR), 767
- P
- PALMES, E. D. (See ALFORD AND PALMES), 1194
- PARÁ, MADUREIRA. The application of a processed glue as a substitute for glass coverslips in histologic technique, 1294
- PARKER, ERNESTINE R., DUNHAM, VOLCOTT B., AND MACNEAL, WARD J. Resistance of the Melbourne strain of influenza virus to desiccation, 37

- , AND MACNEAL, WARD J. Persistence of influenza virus on the human hand, 121
- PARKINS, PHYLLIS V. (See RATCLIFFE AND PARKINS), 534
- PARPART, ARTHUR K. (See CHASE, LORENZ, PARPART, AND GREGG), 99
- PARRISH, D. B., AND CALDWELL, M. J. Modified Koch pipette for Carr-Price reagent dispenser, 992
- PENNOCK, LAZARUS L. Simplification of the treatment of diabetes, 168
- PERRINE, ISABEL. (See EPSTEIN, FOLEY, PERRINE, AND LEE), 319
- PIFFEYER, CARL, DREISBACH, ROBERT H., AND ROAY, CHARLES C. Therapy of nitrogen by electrolytes affecting the blood volume, 709
- PINCK, BERNARD D., MORTON, GLADYS L., AND MATTICE, MARJORIE R. Effect of certain salts on activity of sulacetamide, 462
- PIPER, BERNARD C. (See DENHOFF AND PIPER), 518
- POTI, EDGAR J., AND ROSS, CHARLES A. The clinical use of phthalylsulfathiazole, 785
- POTTER, HAROLD W., AND BRONSTEIN, LEWIS H. The Waterhouse-Friderichsen syndrome, 703
- POWELL, H. M., AND RICE, R. M. Ineffective penicillin chemotherapy of arthritic rats infected with pleuropneumonia-like organisms, 372
- POWERS, J. (See CO TUI, HOPE, SCHRIFT, AND POWERS), 58
- PRICE, C. W. (See RANDALL AND PRICE), 312
- (See WELCH, PRICE, NIELSEN, AND HUNTER), 809
- PRIESTLEY, JOSEPH B. (See HARRIS AND PRIESTLEY), 815

Q

- QUIGLEY, J. P. (See BRODY AND QUIGLEY), 863

R

- RAAB, W. Cardiotonic substances in the blood and heart muscle in uremia (their nature and action), 715
- RACKER, E., AND ROSE, S. P. "Reverse" typing as an aid in the diagnosis of pneumonia, 1203
- RAMSEY, THOMAS L., FALKENSTEIN, ALFONS P., AND SUJKOWSKI, EUGENE J. The use of the albino rat in the pregnancy hormone test, 419
- RANDALL, W. A., AND PRICE, C. W. A rapid method for estimating the bacteriostatic activity of disinfectants, 312
- RANDOLPH, THERON G. (See MALLERY AND RANDOLPH), 203
- , AND MALLERY, O. T., JR. The effect in vitro of propylene glycol on erythrocytes, 197
- RAPPAPORT, MAURICE B., AND LUISADA, ALDO A. Indirect sphygmomanometry, 638
- RATCLIFFE, HERBERT L., AND PARKINS, PHYLLIS V. On the use of Mallory's photostatic acid hematoxylin for staining intestinal protozoa, 534
- RAY, TITUS B., AND NELSON, TRUMAN. Stabilizing serum for blood typing, 766
- REED, C. I. (See SILVER, STECK, AND REED), 48
- REED, G. B., AND KROPP, BENJAMIN. A simple apparatus for determining peripheral arterial pressure in the rabbit's ear, 214
- REED, RACHAEL K. (See SAPIRSTEIN, REED, AND SOUTHARD), 633
- REICH, CARL, SWIRSKY, MORGAN, AND SMITH, DOROTHY. Sternal bone marrow in the aged, 508
- REIN, CHARLES R. (See BARNARD, ROBERT D.), 1287

- REINER, LEDFOLD. (See FAVORITE, REINER, AND LONDON), 899
- REINHARD, EDWARD H. (See HEMPELMANN, REINHARD, MOORE, BIERRAUNT, AND MOORE), 1020
- REIA, FREDERICK. Note on the selenium method for the determination of non-protein nitrogen, 666
- REPICCI, ANTHONY. (See BRIDGE, THURSTON, AND REPICCI), 936
- RICE, F. A. H. (See YOUNG AND RICE), 439, 735
- RICE, R. M. (See POWELL AND RICE), 372
- RIGDON, R. H. Effect of heparin on phagocytosis, 840
- , AND ALLAN, CHESTER, JR. Aneurysms of the vertebral arteries, a consideration of their etiology, 28
- RIVERS, T. D., AND ELLIOTT, K. A. C. Extreme resistance to insulin stupor in a schizophrenic patient, 55
- ROBERTS, CHARLES S. (See TURNER AND ROBERTS), 81
- ROBERTS, HAROLD K., HELWIG, ELSON B., ELMAN, ROBERT, AND MACBRYDE, CYRIL M. Serial biopsy studies of the effects of estrogens on the liver, 1062
- ROBINSON, MILES H. Apparatus for constant rate intravenous injection of ultra short-acting anesthetics in mice, 296
- ROBY, CHARLES C. (See PFEIFFER, DREISBACH AND ROBY), 709
- ROCKENMACHER, M. (See SHAPIRO, TARLOV, OLIVER, GOLDFARB, BOJAR, KASLOW, AND ROCKENMACHER), 282
- ROE, JOSEPH H. (See COHN AND ROE), 106
- ROGERS, R. J. Subacute bacterial endocarditis confined to a pulmonic valve with malformed leaflets, 825
- ROOK, CHARLES W. (See BROWN, O'BRIEN, AND ROOK), 410
- ROSE, D. L. (See GOTTSCHALL, DEKRUIF, COPE, AND LAURENT), 614
- ROSE, S. P. (See RACKER AND ROSE), 1203
- ROSENBERG, SYLVIA I. (See BIERMAN, SHWARTZMAN, AND ROSENBERG), 454
- ROSENFELD, SAMUEL S., AND ROSENFELD, VILMA W. The reaction of *Xenopus laevis* daudin (the South African clawed frog) to human pregnancy serum, 527
- ROSENFELD, VILMA W. (See ROSENFELD AND ROSENFELD), 527
- ROSENTHAL, L. Seroflucculation with a new group of antirens in some pathologic conditions, 1198
- ROSS, CHARLES A. (See POTI AND ROSS), 785
- ROUTH, JOSEPH L. (See FELLOWS AND ROUTH), 1054
- ROWE, ALBERT P., JR., MCBRIDE, ALICE, AND METTIER, STACY R. A study on the relationship of blood iso-agglutinin titers to total serum protein concentrations, 1156
- RULE, COLTER. A simple auscultatory technique for the estimation of the blood pressure of dogs, 97
- RUSKIN, ARTHUR, AND HERRMANN, GEORGE R. Studies in combined diuretic therapy, 486

S

- SALIT, ELIZABETH POWELL, AND TUTTLE, W. W. The variability of heart rate and blood pressure in selected groups of college and high school students, 1139
- SAND, MACHTELD E. (See SMITH, LAWRENCE W.), 241
- SAPIRSTEIN, LEO A., REED, RACHAEL K., AND SOUTHARD, F. D., JR. Assay of renin substrate by low temperature incubation with renin, 633
- SAVITSKY, NATHAN. (See MADONICK AND SAVITSKY), 542

- SCHEMM, F. R. (See LAYNE AND SCHEMM), 347
- SCHLEICHER, EMIL MARO. Giant orthoehromatic erythroblasts, 127
- SCHMIDT, LILLY. (See CO TUI, HOPE, SCHRIFT, AND POWERS), 58
- SCHNITKER, MAURICE A., AND LENHOFF, CHARLES D. Sulfonamide-resistant gonorrhea treated with urea and sulfonamide by mouth, 889
- SCHREK, ROBERT. A method for mounting gross pathologic specimens in Petri dishes, 91
- SCHRIFT, M. H. (See CO TUI, HOPE, SCHRIFT, AND POWERS), 58
- SCHWARTZ, PAULINE. (See BROWN, WILDER, AND SCHWARTZ), 259
- SCOTT, ELYVN, G., AND FINCH, EDMOND P. JR. A modified test for the detection of nitrate reduction, 404
- SCUTCFIELD, BEECHER L. A method of obtaining compressed air for the small laboratory, 745
- SEELY, DOROTHY DIXON, STOOKEY, PAUL F., AND SHERWOOD, NOBLE P. A study of staphylococcus antitoxin titers in normal human sera, 1162
- SELL, FLOYD. A viewing device for reading Kahn reactions, 752
- SHAPIRO, ARTHUR, TARLOV, I. M., OLIVER, R., GOLDFARB, A. I., BOJAR, S., KASLOW, R., AND ROCKENMACHER, M. Plasma clot tensile strength. II. The effect of some physical factors, anticoagulants, and coagulants, 282
- SHEFTEL, A. G. Determination of total and free cholesterol, 875
- SHERWOOD, NOBLE P. (See SEELY, STOOKEY, AND SHERWOOD), 1162
- SHULMAN, BERNARD H., AND JOHNSON, MARY S. A case of meningitis in a premature infant due to a proteolytic Gram-negative bacillus, 500
- SHWARTZMAN, GREGORY. (See BIERMAN, SHWARTZMAN, AND ROSENBERG), 454
- SIFFERT, ROBERT S., AND KRAUTMAN, BENJAMIN. Cold hemagglutination reactions in tuberculosis, 270
- SILBERSTEIN, JOSEPH K. (See GREIFINGER, AND SILBERSTEIN), 1042
- SILVER, A., STECK, IRVING E., AND REED, C. I. A study of the effects of vitamin D on capillary permeability by the use of the dye T-1824, 48
- SILVERSTEIN, R. M. (See FLORIN AND SILVERSTEIN), 879
- SIMPSON, W. M. (See GOTTSCHALL, DEKRUIF, COPE, AND LAURENT), 614
- SIMONSON, ERNST. (See ENZER, SIMONSON, AND BLANKSTEIN), 63
- SLAYKIN, ALICE E. Quick paraffin method for small biopsies, 74
- SLOBODY, LAWRENCE B. An intradermal test for vitamin C subnutrition, 464
- SMITH, DOROTHY. (See REICH, SWIRSKY, AND SMITH), 508
- SMITH, JANE. (See BECKMAN AND SMITH), 43
- SMITH, LAWRENCE W. Chlorophyll: an experimental study of its water-soluble derivatives. IV. The effect of water-soluble chlorophyll derivatives and other agents upon the growth of fibroblasts in tissue culture, 241
- SMITH, M. S. A homemade mechanical pipette, 872
- SMITH, SEDGWICK E. A new hematocrit tube, 301
- SNAPP, F. E. (See ANNEGERS, SNAPP, IVY, AND ATKINSON), 853
- SNYDER, LAURENCE H., AND DOAN, CHARLES A. Studies in human inheritance. XXV. Is the homozygous form of multiple telangiectasia lethal? 1211
- SOLOMON, ERNEST M. Human serum treatment of atypical pneumonia, 493
- SOPHIAN, LAWRENCE H. A method for the recovery of penicillin from the urine, 769
- SOUTHARD, F. D., JR. (See SAPIRSTEIN, REED, AND SOUTHARD), 633
- SPIES, T. D. (See MOORE, VILTER, MINNICHI, AND SPIES), 1226
- SPITZ, SAMUEL H. (See LOUGHLIN, BENNETT, FLANAGAN, AND SPITZ), 921
- SPRAY, ROBB SPALDING. Blood agar plates, 538
- STARE, F. J. (See MCKIBBIN, THAYER, AND STARE), 1109
- STECK, IRVING E. (See SILVER, STECK, AND REED), 48
- STEIN, L. (See WERTHEIMER AND STEIN), 1082
- STEINBERG, BERNHARD, AND DIETZ, ALBERT. Manual and mechanical resuscitation in experimental asphyxia, 695
- STEINER, GABRIEL, AND STEINER, GRETE. New simple silver stain for demonstration of bacteria, spirochetes, and fungi in sections from paraffin-embedded tissue blocks, 868
- STEINER, GRETE. (See STEINER AND STEINER), 868
- STOOKEY, PAUL F. (See SEELY, STOOKEY, AND SHERWOOD), 1162
- SUJKOWSKI, EUGENE J. (See RAMSEY, FALKENSTEIN, AND SUJKOWSKI), 419
- SWAN, LAWRENCE L. The macroscopic determination of blood groups A and B, 763
- SWEENEY, BEATRICE M. The effect of estrone on anaerobic glycolysis of the uterus of the rat in vitro, 957
- SWIRSKY, MORGAN. (See REICH, SWIRSKY, AND SMITH), 508

T

- TAKACS, WILLIAM S. (See TOOMEY, LEWIS, AVERILL, DRURY, AND TAKACS), 21
- TARLOV, I. M. (See SHAPIRO, TARLOV, OLIVER, GOLDFARB, BOJAR, KASLOW, AND ROCKENMACHER), 282
- TAUSSIG, ALBERT E. On the persistence of falsely positive serologic tests for syphilis in nonsyphilitic infections, 473
- TAYLOR, A. CECIL. Apparatus for the freezing-drying of tissues for storage, 657
- TAYLOR, CHARLES B. (See HEMINGWAY AND TAYLOR), 987
- THAYER, S. (See MCKIBBIN, THAYER, AND STARE), 1109
- THURSTON, ARELYN. (See BRIDGE, THURSTON, AND REPICCI), 936
- TOBIE, WALTER C., AND ORR, MARTHA L. Determination of phenol coefficients in presence of surface tension depressants, 767
- TOBIN, CHARLES E. Effect of gonads and adrenals on the absorption of subcutaneous sesame oil, 850
- TOOMEY, JOHN A., LEWIS, NORMAN, AVERILL, EARL, DRURY, WALTER, AND TAKACS, WILLIAM S. Active and passive immunity in experimental hemophilus pertussis infection in mice, 21
- TRASOFF, ABRAHAM, AND MERANZE, DAVID R. The association of acute interstitial pancreatitis with acute pneumococcal mural endocarditis, 590
- TURNER, WILLIAM J., AND ROBERTS, CHARLES S. An adhesive nondrying electrode paste, 81
- TUTTLE, W. W. (See SALIT AND TUTTLE), 1139
- , AND FITTS, LUCILLE. The effect of heat produced by short wave diathermy on the activity of muscle, 609

V

- VAN LOON, EDWARD J., AND CLARK, BYRON B. Observations on the hematologic actions of acetanilid and acetophenetidin in the dog, 942

- VAUGHAN, WARREN T., JR. (See CHAPPLE AND VAUGHAN), 1
 VILTER, R. (See MOORE, VILTER, MINNICH, AND SRIES), 1226
 VOGEL, MILDRED. (See MCGAVACK AND VOGEL), 1256

W

- WAINE, HANS, BAUER, WALTER, AND BENNETT, GRANVILLE A. The effect of subcutaneous injection of urine and urinary extracts from rheumatoid patients into rats, 19
 WAKIM, K. G., AND GATCH, W. D. A cannula for measuring intrathoracic pressure, 984
 WALKER, HARRY. (See BLOOM AND WALKER), 595
 WALLEN, ARTHUR (See Co Tui, HOPE, SCHRIFF, AND POWERS), 58
 WATTS, G. W. T. Treatment and control of epidermophytosis and bromidosis in a state school with cadmium chloride-arsenol solution, 592
 WEISS, R. A device for the protection of microtomic sections, 525
 WELCH, HENRY, PRICE, CLIFFORD W., NIELSEN, JEAN K., AND HUNTER, ALBERT C. The acute toxicity of commercial penicillin, 809
 WERTHEIMER, E., AND STEIN, L. The cold-susceptible globulin fraction of pathologic sera, 1082
 WHITNEY, DAVID V., III. (See COPLEY AND WHITNEY), 117
 WICK, HARRY J., AND LEIGHTY, JOHN A. Rabbit box, 303
 WILBUR, KARL AL. (See COLLIER AND WILBUR), 1123

- WILDE, WALTER S. A micro-decanter for centrifuge washing, 881
 WILDER, VIOLET M. (See BROWN, WILDER, AND SCHWARTZ), 259
 WILLIAMS, ROBERT H., JANDORF, BERNARD J., AND KAY, GLORIA A. Methods for the determination of thioracil in tissues and body fluids, 329
 WILSON, CHARLES S. (See YEAGER AND WILSON), 177
 WILSON, MARY E. (See HOLMES AND WILSON), 1090
 WITLIN, BERNARD. Self-buffering staining solutions, 1097
 WRIGHT, DUWARD O. (See KINSEY AND WRIGHT), 188

Y

- YEAGER, J. FRANKLIN, AND WILSON, CHARLES S. Circa 42, a new itch remedy, 177
 YONKMAN, FREDRICK F. Toxicity of yohimbine hydrochloride, 1222
 —. (See JACOBS AND YONKMAN), 1217
 YOUNG, E. GORDON, AND RICE, F. A. H. Leucocytosis as an index of pyrogenicity in fluids for intravenous use, 735
 —, AND —. On the occurrence of arsenic in human hair and its medicolegal significance, 489
 YOUNG, VIOLA MAE. (See FELSENFELD AND YOUNG), 375

Z

- ZIMMERMAN, S. L. Transient T-wave inversion following paroxysmal tachycardia, 598

SUBJECT INDEX

Book reviews are indicated by (B. Rev.) after the page number.

A

- Absorption of subcutaneous sesame oil, effect of gonads and adrenals on, 850
of sulfonamides from gastrointestinal tract of albino rats, 921
- Acetanilid and acetophenetidin, hematologic actions of, in dog, 942
- Acetophenetidin and acetanilid, hematologic actions of, in dog, 942
- Acid, ascorbic, saturation test, 324
- xylene sulfonic, as protein precipitant for sulfonamide determination in blood, 879
- Acridine compounds, antibacterial effects of, 134, 1177
- Actinomycosis, 815
- Acute agranulocytosis during sulfamerazine therapy, 899
- interstitial pancreatitis, association of, with acute pneumococcal mural endocarditis, 590
- macrocytic hemolytic anemia following administration of sulfadiazine, 347
- pneumococcal mural endocarditis, association of, with acute interstitial pancreatitis, 590
- toxicity of commercial penicillin, 809
- Adrenals and gonads, effect of, on absorption of subcutaneous sesame oil, 850
- Aerobic and anaerobic cultures of wounds, gentian violet-blood agar plates used in, 1090
- Aerosol, adjuvant effect of, upon germicidal action of cadmium chloride, 689
- Aged, sternal bone marrow in, 508
- Agglutination, colloidal particle, microscopic test, 976
- Agranulocytosis, therapy, 899
- Alr, compressed, method of obtaining, for small laboratory, 745
- Albino rat, use of, in pregnancy hormone test, 419
- Alcohol in blood, micro-method for determination of, 429
- isopropyl, acute and chronic toxicity, 561
- Allergic factor in altitude sickness, 831
- Allergy from timbó (Lonchocarpus H.B.K.), 939
- handbook of, 886 (B. Rev.)
- Alloxan diabetes in dogs, 510
- Altitude sickness, possible allergic factor in, 831
- Amino acid nitrogen in blood, micro-determination of, with spectrophotometer and with optical colorimeter, 222
- acids and protein, ingestion of large quantities of, 963
- Anemia in urine, determination of, 447
- Anaerobic and aerobic cultures of wounds, gentian violet-blood agar plates used in, 1090
- glycolysis of uterus of rat in vitro, effect of estrone on, 957
- Anaphylactic shock in actively sensitized guinea pigs, effect of artificially induced fever on, 614
- Anemia, hemolytic, following administration of sulfadiazine, 347
- in patients with pellagra or vitamin B complex deficiency, 1226
- Anesthesia, ether, effects of, upon total erythrocyte and white cell counts of adult female rats, 1070
- Anesthetics, local, in transdermal therapy, deaths following use of, 478
- ultra short-acting, apparatus for constant rate intravenous injection of, in mice, 296
- Aneurysms of vertebral arteries: consideration of their etiology, 28
- Animal experiments, simple ventilation recorder for, 1205
- inoculation and microscopic examination, 75

- Antibacterial effects of acridine compounds, 134, 1177
- Antigenic properties and chemical composition of polysaccharide fraction of three-hour cultures of *Staphylococcus aureus*, 1054
- with new group of conditions, 1198
- lysolecithin and, 1123
- Antiseptic, intestinal, value of sulfathiazole as, 794
- Antitoxin, *Staphylococcus*, titers in normal human sera, 1162
- Aortitis, syphilitic, myocarditis, hepatic cirrhosis, general considerations on pathogenesis, 139
- Apparatus for constant rate intravenous injection of ultra short-acting anesthetics in mice, 296
- for determining peripheral arterial pressure in rabbit's ear, 214
- for freezing-drying of tissues for storage, 657
- for periodic testing of *P. notatum* flask cultures, 749
- for sterile lyophilization of small amounts of material, 978
- Application of "inductograph" to registration of movements, particularly of body structures such as pyloric sphincter, 863
- Applicator, iodine, mechanical, 664
- Arsenic, occurrence of, in human hair and medicolegal significance, 439
- Arterial pressure in rabbit's ear, simple apparatus for determining, 214
- Arteries, vertebral, aneurysms of; consideration of their etiology, 28
- Arthritic rats infected like organism, chemotherapy of, 372
- Ascorbic acid saturation test, 324
- Asphyxia, experimental, manual and mechanical resuscitation in, 695
- Aspirin hypersensitivity, nodal rhythm and bundle branch block following, 595
- Assay method, convenient, for penicillin and similar substances, 310
- of renin substrate by low temperature incubation with renin, 633
- Atabrine, serum, concentration, simple method for estimating, 1303
- Athlete's foot, treatment of, cotton hose as vehicle for fungicide in, 606
- Atypical pneumonia, human serum treatment of, 493
- Auscultatory technique, simple, for estimation of blood pressure of dogs, 97
- Autograft, traumatic, of splenic tissue in body wall, 247
- Automatic pipette, simple, 747
- Autopsy, 230 (B. Rev.)
- Avian malaria infections, apparent advantage of frequently administered quinine in, 43
- Azure A and toluidine blue reactions, standardization and assay of heparin by (correction), 117

B

- Bacillus, proteolytic Gram-negative, meningitis in premature infant due to, 500
- Bacteria, spirochetes, and fungi in sections from paraffin-embedded tissue blocks, silver stain for demonstration of, 868
- Bactericidal activity of some di-(hydroxy)-ectants, rapid, 312
- Bacteriostatic, isolation of, from sputum of atypical case of human tularemia, 903
- typhosus, properties for, infection, 916

Barbiturate intoxication and picrotoxin treatment, 265

Beets, home-canned, botulism from, 702

Bile acids, effect of, on biliary excretion of neoparsphenamine and napharsen, 853

Biliary excretion of neoparsphenamine and napharsen, effect of bile acids on, 853

Biochemistry for medical students, 459 (B. Rev.)

Biopsies, small, quick, paraffin method for, 74

Biopsy studies of effects of estrogens on liver, 1062

Biuret method, direct, for determination of serum proteins, adaptation of, to box comparator colorimetry, 436

Blood agar-gentian violet plates used in aerobic and anaerobic cultures of wounds, 1090

agar plates, 538

amino acid nitrogen in, microdetermination of, with spectrophotometer and with optical colorimeter, 222

and heart muscle in uremia, cardiotoxic substances in, 715

and organs of dogs, reactions in, on intravenous injection of hemoglobin solution, 628

anthelmolytic value of, lysolecithin and, 1123

creatinine, elevated, prognostic significance of, 1001

ethyl alcohol in, micromethod for determination of, 429

films, Romanovsky staining of, and role of methylene violet, 1181

glucose, microdetermination of, 113

groups A and B, macroscopic determination of, 763

in tuberculosis, clinical significance of, 118 (B. Rev.)

iso-agglutinin titers, relationship of, to total serum protein concentration, 1156

pressure and heart rate, variability of, in selected groups of college and high school students, 1139

estimation of, 638

of dogs, estimation of, simple auscultatory technique for, 97

serum, determination of tocopherol in, 772

specific gravity and blood volume, relationship between, in recovery from car-stored, 99

sulfonamide determination in, using xylene-sulfonic acid as protein precipitant, 879

typing, stabilizing serum for, 766

volume and blood specific gravity, relationship between, in recovery from cardiac decompensation, 390

method for determination of, with dye T-1824, 1266

therapy of migraine by electrolytes affecting, 709

Body and facial prosthesis in relation to war wounds, 667

fluids, thionin in, methods for determination of, 329

structures, application of "inductograph" to registration of movements of, 863

Bone chips, grafts, repair of cranial defects, 546

marrow in the aged, 508

Book reviews and notices, 118, 230, 450, 560, 782, 883, 998, 1107, 1210, 1307

Borderlands of psychiatry, 119 (B. Rev.)

Botulism from home-canned beets, 702

Box comparator colorimetry, adaptation of direct biuret method to, for determination of serum proteins, 436

rabbit, 303

Brain pathology, patients with, and normal subjects, reappearance of flicker at high flash frequency in, 63

Brains, color photography of, after fixation, 997

Bromidrosis, cadmium-aerosol solution for, 694

Bundle branch block and nodal rhythm following aspirin hypersensitivity, 595

C

Cadmium chloride, germicidal action of, adjuvant effect of aerosol upon, 649

Cancer, riddle of, 883 (B. Rev.)

Canine distemper virus, natural occurrence of tularemia in dogs used as source of, 966

Cannula for measuring intrathoracic pressure, 884

Capillary permeability, effects of vitamin D on, by use of dye T-1824, 48

Cardiac decompensation, relationship between blood volume and blood specific gravity in recovery from, 390

Cardiotoxic substances in blood and heart muscle in uremia, 715

Care of animals, 80

of human sperm, 211

Carr-Price reagent dispenser, modified Koch pipette for, 992

Cast chip-bone grafts, repair of cranial defects by, 546

Cell growth in vitro, role of creatine in, and its use in wound healing, 483

white, and total erythrocyte counts of adult female rats, effects of ether anesthesia upon, 1070

Centrifuge washing

Chemical composition of : : : : :
three-hour : : : : :
cocci at : : : : :
18, 454
36, of ar-
th pleuro-
372

Chemotherapeutic sulfa drugs upon growth of : : : : :
18, 454
36, of ar-
th pleuro-
372

Chicago area, occurrence of members of genus *Salmonella* in inhabitants of state hospitals of, 375

Chick, P. Iophurac in, effect of heparin on, 840

Children, organ weights of, nomogram for, 1300

Chip-bone grafts, repair of cranial defects by, 546

Chlorophyll, study of its water-soluble derivatives, 441

Cholesterol, total and free, determination of, 875

Choline deficiency studies in dogs, 1109

Circa 42, new itch remedy, 177

Cirrhosis, hepatic, syphilitic aortitis, myocarditis, general considerations on pathogenesis, 139

Citrate utilization, medium for determination of, by colon bacteria, 306

Clinical method for studying factor of human relations in disease, 1

use of oral thermometers, 179

of phthalylsulfathiazole, 785

Clot, plasma, tensile strength of, 282

Cold agglutination, vasospastic factor in serum of case of Raynaud's disease with, 1074

agglutinins in tuberculosis, 936

fraction, 1082

hemagglutination reactions in tuberculosis, 370

Cold-susceptible globulin fraction of pathologic sera, 1082

Colloidal particle agglutination in protein-antiprotein systems, 352

Colon bacteria, medium for determination of citrate utilization by, 306

surgery of, phthalylsulfathiazole as adjuvant in, 798

Color, photographs of fluorescence phenomena in, inexpensive method of publishing, 1207

photography of fixed pathologic specimens, 994

plates, 263, 470, 996, 1094, 1208, 1212

Colorimeter, optical, and spectrophotometer, of amino acid with, 222

of traces of metals,

Colorimetry, box comparator, adaptation of direct biuret method for determination of serum proteins to, 436

Commercial penicillin, acute toxicity of, 809

Comparative results with transdermal (or transcutaneous) and intracutaneous tuberculin tests, 398

Complete pediatrician, 560 (B. Rev.)

- Compounds, acridine, antibacterial effects of, 134, 1177
 gold, in rats, toxicity of, 1168
 Coronary disease associated with short PR interval and prolonged QRS, 673
 Correspondence, 783
 Cotton hose as vehicle for fungicide in treatment of athlete's foot, 606
 Coverslips, glass, processed gluc as substitute for, in histologic technique, 1294
 Cranial defects, repair of, by cast chip-bone grafts, 546
 Creatine, role of, in cell growth in vitro and its use in wound healing, 483
 Creatinine, blood, elevated, prognostic significance of, 1001
 Cultivation and preservation of diplococcus pneumoniae and mycobacterium tuberculosis in market eggs, 532
 Culture media, bacteriologic maintenance of moisture in, 751
 time and temperature guide for inspissating, 526
 Cultures, aerobic and anaerobic, of wounds, gentian violet-blue agar plates used in, 1090
 gonococcus, delayed, solid medium for transportation of, 314
 P. notatum, simple apparatus for periodic testing of, 749

D

- Dark-field diagnosis of penile lesions, 82
 Death following use of local anesthetics in transcoelid therapy, 478
 Decompensation, cardiac, relationship between blood volume and blood specific gravity in recovery from, 390
 Depressants, surface tension, determination of phenol coefficients in presence of, 767
 Desiccation, resistance of Melbourne strain of influenza virus to, 37
 Detection of nitrate reduction, modified test for, 404
 Device for protection of microtome sections, 525
 mechanical, for making multiple paraffin rings for slide precipitation tests for syphilis, 1100
 restraining, for hematological work with rats and mice, 981
 viewing, for reading Kahn reactions, 752
 Kahn, with standard illumination for reading tubes in rack, 755
 Diabetes, alloxan, in dogs, 510
 juvenile, insulin sensitivity, 366
 treatment of, simplification, 168
 Diabetic life, control by diet and insulin, 886 (B. Rev.)
 Diagnosis and treatment of epidemic cerebrospinal meningitis, 585
 dark-field, of penile lesions, 82
 laboratory, of tuberculosis, 75
 medical, 1210 (B. Rev.)
 of malaria, laboratory aids in, 518
 of pneumonia, "reverse" typing as aid in, 1203
 of tularemia, rapid agglutination test for, 976
 Diarrheas, treatment of, with phthalylsulfathiazole, 796
 Diathermy, short wave, effect of heat produced by, on activity of muscle, 609
 Diet and insulin, control by, in diabetes, 886 (B. Rev.)
 Diethylstilbestrol, chemical and morphologic responses to, 1062
 Diets, elimination, and patient's allergies, 886 (B. Rev.)
 "Digestion" of mictal in stomach, 687
 Di-(hydroxyphenyl) alkanes, bactericidal activity of some, 254
 Dogs, alloxan diabetes in, 510
 blood pressure of, simple auscultatory technique for estimation of, 97
 choline deficiency studies in, 1109
 hematologic actions of acetanilid and acetophenetidin in, 942
 natural occurrence of tularemia in, used as source of canine distemper virus, 906
 reactions in blood and organs of, on intravenous injection of hemoglobin solution, 628

- Diplococcus pneumoniae and mycobacterium tuberculosis, cultivation and preservation of, in market eggs, 532
 Disease, coronary, associated with short PR interval and prolonged QRS, 673
 creatinine content of blood in, 1004
 human relations in, clinical method for studying, 1
 hypertensive, and hypertension, 998 (B. Rev.)
 Raynaud's, with cold agglutination, vasospastic factor in serum of case of, 1074
 Diseases, heart, 999 (B. Rev.)
 Disinfectants, bacteriostatic activity of, rapid method for estimating, 312
 Dispenser, Carr-Price reagent, modified Koch pipette for, 992
 Distemper virus, natural occurrence of tularemia in dogs used as source of, 906
 Diuretic therapy, combined, studies in, 486
 Drop recorder, electron tube, 410
 Drugs, sulfa, effect of, upon growth of a-hemolytic Streptococcus, 454
 Dye T-1824, effects of vitamin D on capillary permeability by use of, 48
 method for determination of blood volume with, 1266

E

- EA-1, pharmacologic action of, 150
 Eberthella typhosa, purified pyrogen from, 58
 Editorial policy, 781
 Eggs, cultivation and preservation of diplococcus pneumoniae and mycobacterium tuberculosis in, 532
 Electrode paste, adhesive nondrying, 81
 Electrolytes affecting blood volume, therapy of migraine by, 709
 Electron tube drop recorder, simple, 410
 Elliptocytosis, 185
 Endocarditis, pneumococcal mural, association of, with acute interstitial pancreatitis, 590
 subacute bacterial, confined to pulmonary valve with malformed leaflets, 825
 Eosinophilia, local, in malignant neoplasms, 820
 Epidemic cerebrospinal meningitis, diagnosis and treatment of, 585
 Erythroblasts, giant orthochromatic, 127
 Erythrocyte counts of adult female rats, effects of ether anesthesia upon, 1070
 Erythrocytes, clumping of, in Hayem's diluting fluid, grave prognostic sign, 624
 effect in vitro of propylene glycol on, 197
 reversibility of sensitization of, 684
 Estradiol, chemical and morphologic responses to, 1062
 Estrogens, serial biopsy studies of effects of, on liver, 1062
 Estrone, effect of, on anaerobic glycolysis of uterus of rat in vitro, 957
 Ether anesthesia, effects of, upon total erythrocyte and white cell counts of adult female rats, 1070
 Ethyl alcohol in blood, micromethod for determination of, 429
 Experimental hypertension, sympatholytic treatment of, 1217
 Experiments, animal, simple ventilation recorder for, 1205
 Extracts, tissue, micro-mincer for preparation of, 1104
 Extremities of man, vascular responses in, in health and disease, 782 (B. Rev.)
 Eyes, color photography of, after fixation, 996

F

- Facial and body prosthesis in relation to war wounds, 667
 Faeces of chronic invalids, plasma-coagulating staphylococci in, 938
 Fever, artificially induced, effect of, on anaphylactic shock in actively sensitized guinea pigs, 614
 Fibroblasts in tissue culture, growth of, effect of water-soluble chlorophyll derivatives and other agents upon, 241
 Flicker, reappearance of, at high flash frequency in patients with brain pathology and in normal subjects, 63

Fluids for intravenous use, leucocytosis as index of pyogenicity in, 735
 Fluorescence microscopy, staining tubercle bacilli in, comparison of procedures for, 218
 phenomena, photographs of, in color, inexpensive method of publishing, 1207
 Food infection, *Salmonella*, in military personnel, 1042
 Fraction, globulin, cold-susceptible, of pathologic sera, 1082
 polysaccharide, of three-hour cultures of *Staphylococcus aureus*, chemical composition and antigenic properties of, 1054
 Freezing-drying of tissues for storage, apparatus for, 657
 Frog, South African clawed, reaction of, to human pregnancy serum, 527
 Fungi, bacteria, and spirochetes in sections from paraffin-embedded tissue blocks, silver stain for demonstration of, 868
 Fungicide, cotton hose as vehicle for, in treatment of athlete's foot, 606

G

Gastric mucus smears, permanent metachromatic staining of, 321
 Gentian violet-blood agar plates used in aerobic and anaerobic cultures of wounds, 1090
 Germicidal action of cadmium chloride, adjuvant effect of aerosol upon, 689
 Germicidal action of aerosol upon, method to 127
 Giant cell, 127
 Glass, substitute 1294
 for, in histologic technique, 1294
 Glucose, blood microdetermination of, 113
 Glue, processed as substitute for glass coverslips in histologic technique, 1294
 Glycogen metabolism in liver under fasting conditions, 106
 Glycogenesis, new technique for measurement of, 106
 Gold compounds in rats, toxicity of, 1168
 reaction, significance of pH in, 970
 Gonads and adrenals, effect of, on absorption of subcutaneous sesame oil, 850
 Gonococcus cultures, delayed, solid medium for transportation of, 314
 Gonorrhea, sulfonamide-resistant, treated with urea and sulfonamide by mouth, 889
 Grafts, cast chip-bone, repair of cranial defects by, 646
 Guide, time and temperature, for inspissating culture media, 526
 Guinea pigs, actively sensitized, anaphylactic shock in, effect of artificially induced fever on, 614

H

Hair, human, occurrence of arsenic in, and medicolegal significance, 439
 Hand, influenza virus on, persistence of, 121
 Hayen's diluting fluid, clumping of erythrocytes in, grave prognostic sign, 624
 Head injuries, 68
 Heart diseases, 999 (B. Rev.)
 muscle and blood in uremia, cardiotoxic substances in, 715
 rate and blood pressure, variability of, in selected groups of college and high school students, 1139
 Heat produced by short wave diathermy, effect of, on activity of muscle, 609
 Hematocrit tube, new, 301
 Hematologic actions of acetanilid and acetophenetidin in dog, 942
 complications of therapy with radioactive phosphorus, 1020
 work, improved restraining device for rats and mice, 981
 Hemochromatosis, mobilization of iron in, with administration of various chemicals, 742
 Hemoglobin solution, intravenous injection of, reactions in blood and organs of dogs on, 628
 Hemolysis, per cent, methemoglobin-oxyhemoglobin ratio and, in stored blood, method for simultaneous determination of, 99

a-Hemolytic *Streptococcus*, effect of chemotherapeutic sulfa drugs upon growth of, 164
 Hemophilus pertussis infection in mice, active and passive immunity in, 21
 Hemorrhage, bilateral adrenal (Waterhouse-Friderichsen syndrome) associated with meningococcal septicemia, 357
 Heparin, effect of, on phagocytosis, 840
 standardization and assay of, by toluidine blue and azure A reactions (coirrection), 117
 Hepatic cirrhosis, syphilitic aortitis, myocarditis, general considerations on pathogenesis, 139
 Hippuric acid excretion test, reaction following, 188
 Histologic technique, application of processed glue as substitute for glass coverslips in, 1294
 Holder, multiple rabbit, 308
 Home-canned beets, botulism from, 702
 Homemade mechanical pipette, 872
 Homicide investigation, 885 (B. Rev.)
 Hormones, fat-soluble, vehicle for intravenous administration of, 530
 steroid, propylene glycol as menstruum for administration of, 1256
 Hose, cotton, as vehicle for fungicide in treatment of athlete's foot, 606
 Human hair, occurrence of arsenic in, and hand, p on, 121
 infant, of, 230
 (B. Rev.)
 inheritance, studies in, 1211
 plasma, recalcified, serum from, suitability of, for serodiagnostic tests for syphilis, 1287
 pregnancy serum, reaction of *Xenopus laevis* daudin (South African clawed frog) to, 527
 relations in disease, clinical method for studying factor of, 1
 sera, normal *Staphylococcus antitoxin* titers in, 1162
 serum treatment of atypical pneumonia, 493
 sperm, care of, 211
 Humidity and temperature, effect of, on oxygen toxicity, 1134
 Hypertension and hypertensive disease, 998 (B. Rev.)
 experimental, sympatholytic treatment of, 1217

I

Immunity, active and passive, in experimental *Hemophilus pertussis* infection in mice, 21
 Implantation, intraperitoneal, of reconstituted desiccated muscle, shock from, 339
 Indirect sphygmomanometry, 638
 "Inductograph," application of, to registration of movements, particularly of body structures such as pyloric sphincter, 863
 Infant, neuromuscular maturation of, 230 (B. Rev.)
 premature, meningitis in, due to proteolytic Gram-negative bacillus, 500
 Infection, experimental *Hemophilus pertussis*, active and passive immunity in, in mice, 21
 food, *Salmonella*, in military personnel, 1042
 Infections, malaria, avian, apparent advantage of frequently administered quinine in, 13
 meningococcal purpuric lesions in, 273
 nonsyphilitic, persistence of falsely positive serologic tests for syphilis in, 473
 Influenza virus on human hand, persistence of, 121
 resistance of Melbourne strain of, to desiccation, 37
 Ingestion of large quantities of protein and amino acids, 963
 Inhalational therapy, principles and practices of, 887 (B. Rev.)
 Inheritance, human, studies in, 1211
 Injection, intramuscular, oils used for, 259
 intravenous, of hemoglobin solution, reactions in blood and organs of dogs on, 628
 of ultra short-acting anesthetics in mice, 296

Injection—Cont'd

- subcutaneous, of urine and urinary extracts from rheumatoid patients into rats, 19
- Insulin and diet, control by, in diabetes, 886 (B. Rev.)
- in diabetes, 172
- sensitivity, juvenile diabetes, 366
- stupor in schizophrenic patient, extreme resistance to, 55
- Intestinal antiseptic, value of sulfathiazole as, 794
- protozoa, Mallory's phosphotungstic acid hematoxylin for staining, 534
- Intoxication, barbiturate, picrotoxin treatment and, 265
- Intracutaneous and transdermal tuberculin tests, comparative results with, 393
- Intradermal test for vitamin C subnutrition, 464
- Intramuscular injections, oils used for, 259
- Intrathoracic pressure, cannula for measuring, 984
- Intravenous administration of fat-soluble hormones, vehicle for, 530
- injection of hemoglobin solution, reactions in blood and organs of dogs on, 628
- use, fluids for, leucocytosis as index of pyrogenicity in, 735
- Investigation, homicide, 885 (B. Rev.)
- Iodine applicator, mechanical, 664
- Iron, mobilization of, in hemochromatosis, with administration of various chemicals, 742
- Isopropyl alcohol, acute and chronic toxicity of, 561
- Itch remedy, Circa 42, 177

J

- Juvenile diabetes insulin sensitivity, 366

K

- Kahn reactions, viewing device for reading, 752
- shaker, paraffin tissue preparations utilizing, 304
- viewing device with standard illumination for reading tubes in rack, 755
- Koch pipette, modified, for Carr-Price reagent dispenser, 992

L

- Laboratory aids in diagnosis of malaria, 518
- diagnosis of tuberculosis, 75
- polychrome stain for use in, 1094
- small, method of obtaining compressed air for, 745
- supports, 536
- tests of oximeter with automatic compensation for vasomotor changes, 987
- Lesions, penile, dark-field diagnosis of, 82
- purpuric, in meningococcal infections, 273
- Leucocytes, effect in vitro of propylene glycol on, 203
- Leucocytosis as index of pyrogenicity in fluids for intravenous use, 735
- Liver damage, severe, reaction following ingestion of sodium benzoate in patient with, 188
- glycogen metabolism in, under fasting conditions, 106
- serial biopsy studies of effects of estrogens on, 1062
- Local anesthetics in transdermal therapy, deaths following use of, 478
- eosinophilia in malignant neoplasms, 802
- Lonchocarpus H.B.K. (timbó), allergy from, 939
- Lyophilization, sterile, of small amounts of material, simple inexpensive apparatus for, 978
- Lysocleithin and antihemolytic value of blood, 1123

M

- Macrocytic anemia, nutritional, in patients with pellagra or vitamin B complex deficiency, 1226
- Macroscopic determination of blood groups A and B, 763

- Malaria, diagnosis, laboratory aids in, 518
- treatment, prophylaxis, 1307 (B. Rev.)
- infections, avian, apparent advantage of frequently administered quinine in, 43
- Malarial parasites, simple acid water solution for better visualization of, using Wright's stain for thick blood films, 1103
- Vi agglutinative properties for *Bacterium typhosum* demonstrated following infection with, 916
- Malignant neoplasms, local eosinophilia in, 820
- Mallory's phosphotungstic acid hematoxylin for staining intestinal protozoa, 534
- Manual and mechanical resuscitation in experimental asphyxia, 695
- Mapharsen and neoarsphenamine, biliary excretion of, effect of bile acids on, 853
- Maturation, neuromuscular, of human infant, 230 (B. Rev.)
- Mechanical and manual resuscitation in experimental asphyxia, 695
- device for making multiple paraffin rings for slide precipitation tests for syphilis, 1100
- iodine applicator, 664
- pipette, homemade, 872
- Media, culture, bacteriologic maintenance of moisture in, 751
- time and temperature guide for inspissating, 526
- Medical diagnosis, 1210 (B. Rev.)
- illustration, 546, 667, 994, 1207
- students, biochemistry for, 450 (B. Rev.)
- Medicolegal significance of arsenic in human hair, 439
- Medium for determination of citrate utilization by colon bacteria, 306
- solid, for transportation of delayed gonococcus cultures, 314
- Melbourne strain of influenza virus, resistance of, to desiccation, 37
- Meningitis, epidemic cerebrospinal, diagnosis and treatment of, 585
- in premature infant due to proteolytic Gram-negative bacillus, 500
- Meningococcal septicæmia hemorrhage (sen syndrome)
- Meningococcal infections, purpuric lesions in, 273
- Metabolism, glycogen, in liver under fasting conditions, 106
- Metachromatic staining, permanent, of gastric mucus smears, 321
- Metal, "digestion" of, in stomach, 687
- Metals, traces of, colorimetric determination of, 782 (B. Rev.)
- Methemoglobin-oxyhemoglobin ratio and percent hemolysis in stored blood, method for simultaneous determination of, 99
- Method, assay, for penicillin and similar substances, 319
- clinical, for studying factor of human relations in disease, 1
- for determination of blood volume with dye T-1824, 1266
- of penetratin, 329
- of thiouracil, 329
- for estimating infectants, 312
- serum atabrine concentration, 1303
- for mounting gross pathologic specimens in Petri dishes, 91
- for obtaining compressed air for small laboratory, 745
- for paraffin tissue preparations utilizing Kahn shaker, 304
- for publishing photographs of fluorescence phenomena in color, 1207
- for recovery of penicillin from urine, 769
- for simultaneous determination of methemoglobin-oxyhemoglobin ratio and percent hemolysis in stored blood, 99
- quick paraffin, for small biopsies, 74
- selenium, for determination of nonprotein nitrogen, 666
- 2-Methylaminoheptane (EA-1), pharmacologic action of, 150
- Methylenc violet, role of, in Romanovsky staining of blood films, 1181
- Mice, active and passive immunity in experimental *Hemophilus pertussis* infection in, 21

Mice—Cont'd

- apparatus for constant rate intravenous injection of ultra short-acting anesthetics in, 296
- improved restraining device for hematological work with, 981
- methods of infecting, 21
- Micro-decanter for centrifuge washing, 881
- Microdetermination of blood glucose, 113
- Micromethod for determination of ethyl alcohol in blood, 429
- Micro-mincer to facilitate preparation of tissue extracts, 1104
- Microscopic observation of colloidal particle agglutination in protein-antiprotein systems, 352
- Microscopy, fluorescence, staining tubercle bacilli in, comparison of procedures for, 218
- Microtome sections, device for protection of, 525
- Micrurgical and germ-free techniques, 119 (B. Rev.)
- Migraine, therapy of, by electrolytes affecting blood volume, 709
- Military personnel, Salmonella food infection in, 1042
- Mobilization of iron in hemochromatosis with administration of various chemicals, 742
- Moisture, maintenance of, in bacteriologic culture media, 751
- Mounting gross pathologic specimens in Petri dishes, 91
- Mural endocarditis, pneumococci, association of, with acute interstitial pancreatitis, 590
- Muscle, activity of, effect of heat produced by short wave diathermy on, 609
- desiccated, reconstituted, shock from intraperitoneal implantation of, 339
- Mycobacterium tuberculosis and diplococcus

N

- Neonarsphenamine and mapharsen, biliary excretion of, effect of bile acids on, 853
- Neoplasms, malignant, local eosinophilia in, 820
- Neuromuscular maturation of human infant, 230 (B. Rev.)
- Nitrate reduction, modified test for detection of, 404
- Nitrogen, nonprotein, selenium method for determination of, 666
- Nodal rhythm and bundle branch block following aspirin hypersensitivity, 595
- Nomogram for organ weights of children, 1300
- Nutritional macrocytic anemia in patients with pellagra or vitamin B complex deficiency, 1226
- status of Staphylococcus aureus as influenced by proflavine, 383

O

- Oils used for intramuscular injections, 259
- Opium alkaloids, pharmacology of, 450 (B. Rev.)
- Oral thermometers, clinical use of, 179
- Organ weights of children, nomogram for, 1300
- Oscillometer and thermocouple as diagnostic aids in peripheral vascular disease, 1147
- Oximeter with automatic compensation for vasomotor changes, laboratory test of, 987
- Oxygen toxicity, effect of humidity and temperature on, 1134

P

- P. lophurae in chick, effect of heparin on, 840
- P. notatum flask cultures, simple apparatus for periodic testing of, 749
- Pancreatitis, acute interstitial, association of, with acute pneumococci mural endocarditis, 590
- Pandy test and total protein content of spinal fluid, relation between, 542

- Pappenheim stain, stable modification, 207
- Paraffin method, quick, for small biopsies, 74
- Parings, multiple, mechanical device for making, for slide precipitation tests for syphilis, 1100
- tissue preparations utilizing Kahn shaker, simple rapid method for, 304
- Parkinson's disease, 67
- Paroxysmal tachycardia, transient T-wave inversion following, 598
- Paste, electri
- Pathogenesis:
 - ilit
 - ic
- Pathognostic seroagglutination, 1198
- Pathologic conditions, seroagglutination with new group of antigens in, 1198
- sera, cold-susceptible globulin fraction of, 1082
- specimens, fixed color photography of, 991
- Pediatrian, complaint, 560 (B. Rev.)
- Pellagra, patient with, nutritional macrocytic anemia in, 1226
- Penicillin chemotherapy, ineffective, of arthritic rats infected with pleuropneumonia-like organism, 372
- commercial, acute toxicity of, 809
- convenient assay method for, 319
- recovery of, from urine, 769
- Penile lesions, dark-field diagnosis of, 82
- Peripheral arterial pressure in rabbit's ear, simple apparatus for determining, 214
- vascular disease, oscilometer and thermocouple as diagnostic aids in, 1147
- pH in gold reaction, significance of, 970
- Phagocytosis, effect of heparin on, 840
- Pharmacologic action of 2-methylaminoheptane (EA-1), 150
- Pharmacology of opium alkaloids, 450 (B. Rev.)
- Phenol coefficients, determination of, in presence of surface tension depressants, 767
- Phosphorus, radioactive, hematologic complications of therapy with, 1020
- Photographs of fluorescence phenomena in color, inexpensive method for publishing, 1207
- Photography, color, of fixed pathologic specimens, 994
- Phthalylsulfathiazole, clinical use of, 785
- Physical diagnosis, applied, 1210 (B. Rev.)
- Picrotoxin treatment and barbiturate intoxication, 265
- Pipette, automatic, simple, 747
- Koch, modified, for Carr-Price reagent dispenser, 992
- mechanical homemade, 872
- modified, for prothrombin determinations, 986
- Plasma clot tensile strength, 282
- human, recalcified, serum from, for sero-diagnostic tests for syphilis, 1287
- Plasma-coagulating staphylococci in feces of chronic invalids, 938
- Plates, blood agar, 538
- Pleuropneumonia-like organisms, arthritic rats infected with, ineffective penicillin chemotherapy of, 372
- Pneumococcal pneumonia, treatment of, with sulfamethazine, 568
- Pneumococci mural endocarditis, association of, with acute interstitial pancreatitis, 590
- Pneumonia, atypical, human serum treatment of, 493
- pneumococcal, treatment of, with sulfamethazine, 568
- "reverse" typing as aid in diagnosis of, 1203
- Polley, editorial, 781
- stains, 1097
- in clinical
- ilit, 231
- QRS, coronary, 773
- Pregnancy hormone test, use of albino rat in, 419
- in juvenile diabetes, 369
- serum, human, reaction of Xenopus laevis dandini (South African clawed frog) to, 527

- Preservation of, cultivation and, of diplococcus pneumoniae and mycobacterium tuberculosis in market eggs, 532
- Proflavine, nutritional status of Staphylococcus aureus as influenced by, 383
- Promegaloblast, 127
- Pronormoblast, 127
- Propylene glycol as menstruum for administration of steroid hormones, 1256
- effect in vitro of, on erythrocytes, 197
- on leucocytes, 203
- Prosthesis, facial and body, in relation to war wounds, 667
- Protein and amino acids, ingestion of large quantities of, 963
- content of spinal fluid and Pandey test, relation between, 542
- serum, concentration, relationship of blood iso-agglutinin titers to, 1156
- Protein-antiprotein systems, microscopic observation of colloidion particle agglutination in, 352
- Proteins, serum, determination of, adaptation of direct biuret method to box comparator colorimetry, 436
- Proteinuria, simplified and rapid test for, 228
- Prothrombin determinations, modified pipette for, 986
- Psychiatry, borderlands of, 119 (B. Rev.)
- fundamentals of, 884 (B. Rev.)
- Pulmonary disease, chronic, in South Wales coalminers, 119 (B. Rev.)
- Pulmonic valve with malformed leaflets, subacute bacterial endocarditis confined to, 325
- Purpuric lesions in meningococcal infections, 273
- Pyloric sphincter, application of "inductograph" to registration of movements of, 863
- Pyrogen, purified, from Eberthella typhosa, 58
- Pyrogenicity, leucocytosis as index of, in fluids for intravenous use, 735
- Q
- QRS, prolonged, and short PR interval, coronary disease associated with, 673
- Quinine in avian malaria infections, apparent advantage of frequently administered, 43
- R
- Rabbit box, 303
- holder, multiple, 308
- Rabbits, experiments on, Raynaud's disease with cold agglutination, 1074
- kidneys of pathologic, prolonged by pyrazine, in, compared and sulfadiazine, 574
- peripheral arterial pressure in ears of, simple apparatus for, 214
- Rabies, 120 (B. Rev.)
- Radioactive phosphorus, hematologic complications of therapy with, 1020
- Rats, adult female, effects of ether anesthesia upon total erythrocyte and white cell counts of, 1070
- albino, absorption of sulfonamides from gastrointestinal tract of, 921
- use of, in pregnancy hormone test, 419
- arthritic, infected with pleuropneumonia-like organisms, ineffective penicillin chemotherapy of, 372
- effect of estrone on anaerobic glycolysis of uterus of, 957
- of subcutaneous injection of urine and urinary extracts from rheumatoid patients into, 19
- improved restraining device for hematological work with, 981
- toxicity of gold compounds in, 1168
- Raynaud's disease with cold agglutination, vasospastic factor in serum of case of, 1074
- Reaction following ingestion of sodium benzoate in patient with severe liver damage, 188
- of *Xenopus laevis* daudin (South African clawed frog) to human pregnancy serum, 527
- Reactions, cold hemagglutination, in tuberculosis, 270
- in blood and organs of dogs on intravenous injection of hemoglobin solution, 628
- Kahn, viewing device for, 752
- Recorder, drop, electronic tube, 410
- ventilation, simple, for animal experiments, 1205
- Relationship between blood volume and blood specific gravity in recovery from cardiac decompensation, 390
- Pandy test and total protein content of spinal fluid, 542
- Remedy, itch, Circa 42, 177
- Renin, low temperature incubation with, assay of renin substrate by, 633
- substrate, assay of, by low temperature incubation with renin, 633
- Repair of cranial defects by cast chip-bone grafts, 546
- Restraining device, improved, for hematological work with rats and mice, 981
- Resuscitation, manual and mechanical, in experimental asphyxia, 695
- Rheumatoid patients, effect of subcutaneous injection of urine and urinary extracts from, into rats, 19
- Romanovsky staining of blood films, and role of methylene violet, 1181
- S
- Salmonella anatum, 1042
- food infection in military personnel, 1042
- oranienburg, 1042
- types, occurrence of, in inhabitants of state hospitals of greater Chicago area, 375
- typhi murium, 1042
- Salts, effect of, on activity of sulfacetamide, 462
- Saturation test, ascorbic acid, 324
- Schizophrenic patient, insulin stupor in, extreme resistance to, 55
- Selenium method for determination of non-protein nitrogen, 666
- Sensitization of erythrocytes, reversibility of, 684
- Septicemia, meningococcal hemorrhagic (sen syndrome)
- Sera, human, normal, staphylococcus antitoxin titers in, 1162
- Seroflocculation with new group of antigens in some pathologic conditions, 1198
- Serum atabrine concentration, simple method for estimating, 1303
- blood, determination of tocopherol in, 772
- from recalcified human plasma, suitability of, for serologic test for syphilis, 1287
- of case of Raynaud's disease with cold agglutination, vasospastic factor in, 1074
- protein concentration, relationship of, to titers, 1156
- proteins adaptation of direct biuret method to box comparator colorimetry, 436
- stabilizing, for blood typing, 766
- treatment, human, of atypical pneumonia, 493
- Sesame oil, effect of gonads and adrenals on absorption of, 850
- Shock, anaphylactic, in actively sensitized guinea pig, effect of artificially induced fever on, 614
- from intraperitoneal implantation of reconstituted desiccated muscle, 339
- patients in, determination of blood volume in, 1273
- Short wave diathermy, effect of heat produced by, on activity of muscle, 609
- Sickness, altitude, possible allergic factor in, 831
- Silver stain for demonstration of bacteria, spirochetes, and fungi in sections from paraffin-embedded tissue blocks, 868
- Smears, gastric mucus, permanent metachromatic staining of, 321
- Sodium benzoate, reaction following ingestion of, in patient with severe liver damage, 188

- Solution, simple acid water, for better visualization of malarial parasites using Wright's stain for thick blood films, 1103
- mease, concentrated, more stable, preparation of, 337
- Solutions, staining, self-buffering, 1027
- South African clawed frog, reaction of, to human pregnancy serum, 527
- South Wales coalminers, chronic pulmonary disease in, 119 (B. Rev.)
- Specimens, fixed pathologic, color photography of, 994
- gross pathologic, method of mounting, in Petri dishes, 91
- Spectrophotometer and optical colorimeter, microdetermination of amino acid nitrogen in blood with, 222
- Sperm, human, care of, 211
- Sphygmomanometer cuff, effect of distal application of, on localized venous pressure and aid in venipuncture, 539
- Sphygmomanometry, indirect, 638
- Spinal fluid, total protein content of, and Pandy test, relation between, 542
- Spirochetes, bacteria, and fungi in sections from paraffin-embedded tissue blocks silver stain for demonstration of, 868
- Splenic tissue, traumatic autograft of, in body wall, 247
- Stain, Mallory's phosphotungstic acid hematoxylin, for intestinal protozoa, 534
- Pappenheim, stable modification, 207
- Polychrome No. 28, for use in clinical laboratory, 1094
- silver, for demonstration of bacteria, spirochetes, and fungi in sections from paraffin-embedded tissue blocks, 868
- Wright's polychrome methylene blue-eosin, 1097
- Staining, metachromatic, permanent, of gastric mucus smears, 321
- and role of
- comp.
- Staphylococci, chron
- Staphylococcus, huma
- aureus, nutritional status of, as influenced by proflavine, 383
- polysaccharide fraction of three-hour cultures of, chemical composition and antigenic properties of, 1054
- Sternal bone marrow in the aged, 508
- Steroid hormones, propylene glycol as menstruum for administration of, 1256
- Stomach, "digestion" of metal in, 687
- Stupor, insulin, in schizophrenic patient, extreme resistance to, 55
- Subacute bacterial endocarditis confined to pulmonic valve with malformed leaflets, 825
- Subnutrition, vitamin C, intradermal test for, 464
- Sulfacetamide, effect of certain salts on activity of, 462
- Sulfadiazine, acute macrocytic hemolytic anemia following administration of, 347
- and sulfathiazole, prolonged administration of sulfapyrazine and sulfamethyldiazine in kidneys of rabbits compared with, 574
- Sulfamer
- therapy, acute agranulocytosis during, 899
- Sulfamethazine treatment of pneumococcal pneumonia, 568
- Sulfamethyldiazine (sulfamerazine) and sulfapyrazine, prolonged administration of, in kidneys of rabbits as compared with sulfathiazole and sulfadiazine, 574
- Sulfapyrazine and sulfamethyldiazine (sulfamerazine), prolonged administration of, in kidneys of rabbits as compared with sulfathiazole and sulfadiazine, 574
- effect of, upon growth of *a-hemolyticus* Streptococcus, 454
- Supports, laboratory, 536
- Surface tension depressants, determination of phenol coefficients in presence of, 767
- Symptoms, analysis and interpretation of, 999 (B. Rev.)
- Syndrome, Waterhouse-Friderichsen, 703
- Syphilis, mechanical device for making multiple paraffin rings for slide precipitation tests for, 1100
- persistence of falsely positive serologic tests for, in nonsyphilitic infections, 473
- serodagnostic tests for, suitability of serum from recalcified human plasma for, 1287
- T
- Tachycardia, paroxysmal, transient T-wave inversion following, 598
- Technique for measurement of glycogenesis, 106
- simple auscultatory, for estimation of blood pressure of dogs, 97
- Techniques, microsurgical and germ-free, 119 (B. Rev.)
- Telangiectasia, multiple, homozygous form of, 1211
- Temperature and humidity, effect of, on oxygen toxicity, 1134
- and time guide for inspissating culture media, 526
- Tensile strength of plasma clot, 282
- Test, agglutination, rapid, for diagnosis of tularemia, 976
- ascorbic acid saturation, 324
- cold autoagglutination, 271
- isoagglutination, 270
- creatinine clearance, 1015
- for pregnancy, reaction of *Xenopus laevis* daudin (South African clawed frog) to human pregnancy serum, 527
- for proteinuria, simplified and rapid, 228
- intradermal, for vitamin C subnutrition, 464
- modified, for detection of nitrate reduction, 404
- Pandy, and total protein content of spinal fluid, relation between, 542
- pregnancy hormone, use of albino rat in, 419
- Testing, periodic, of *P. notatum* flask cultures, simple apparatus for, 749
- splenic, traumatic autograft of, in body wall, 247
- Tests for syphilis, serodiagnostic, suitability of serum from recalcified human plasma for, 1287
- laboratory, of oximeter with automatic compensation for vasomotor changes, 987
- serologic, falsely positive, persistence of, for syphilis in nonsyphilitic infections, 473
- slide precipitation, for syphilis, mechanical device for making multiple paraffin rings for, 1100
- tuberculin, transdermal and intracutaneous, comparative results, 338
- Texto de bacteriologia, 118 (B. Rev.)
- Therapy, diuretic, combined, studies in, 486
- inhalational, principles and practices of, 887 (B. Rev.)
- of migraine by electrolytes affecting blood volume, 709
- sulfamerazine, acute agranulocytosis during, 899
- transcricoid, deaths following use of local anesthetics in, 478
- with radioactive phosphorus, hematologic complications of, 1020
- Thermocouple and oscilometer as diagnostic aids in peripheral vascular disease, 1147
- Thermometers, oral, clinical use of, 179

- Thiouracil in tissues and body fluids, methods for determination of, 329
- Timbó (Lonchocarpus H.B.K.), allergy from, 939
- Time and temperature guide for inspissating culture media, 526
- Tissue blocks, paraffin-embedded, silver stain for demonstration of bacteria, spirochetes, and fungi in sections from, 868
- culture, growth of fibroblasts in, effect of water-soluble chlorophyll derivatives and other agents upon, 241
- extracts, micro-mineer for preparation of, 1104
- Tissues, freezing-drying of, for storage, apparatus for, 657
- thiouracil in, methods for determination of, 329
- Tocopherol, determination of, in blood serum, 772
- Toluidine blue and azure A reactions, standardization and assay of heparin by (correction), 117
- Toxicity, acute and chronic, of isopropyl alcohol, 561
- of commercial penicillin, 809
- of gold compounds in rats, 1168
- of yohimbine hydrochloride, 1222
- oxygen, effect of humidity and temperature on, 1134
- Transcricoid therapy, deaths following use of local anesthetics in, 478
- Transcutaneous and intracutaneous tuberculin tests, comparative results with, 398
- Transdermal and intracutaneous tuberculin tests, comparative results with, 398
- Transportation of delayed gonococcus cultures, solid medium for, 314
- Traumatic autograft of splenic tissue in body wall, 247
- Treatment, human serum, of atypical pneumonia, 493
- of athlete's foot, cotton hose as vehicle for fungicide in, 606
- of diabetes, 168
- of epidemic cerebrospinal meningitis, 585
- of pneumococcal pneumonia with sulfamethazine, 568
- of sulfonamide-resistant gonorrhea with urea and sulfonamide by mouth, 889
- picrotoxin, and barbiturate intoxication, 265
- sympatholytic, of experimental hypertension, 1217
- Treponema pallidum, differential motility characteristics of, 82
- Trichinosis, 1307 (B. Rev.)
- Tube, hematocrit, new, 301
- Tubercle bacilli, comparison of procedures for staining, in fluorescence microscopy, 218
- bacillus, polysaccharide from, 231
- Tuberculin tests, transdermal and intracutaneous, comparative results, 398
- Tuberculosis, blood in, clinical significance of, 118 (B. Rev.)
- reactions in, 270
- of, 75
- Tularemia, diagnosis of, rapid agglutination test for, 976
- human, isolation of *Bacterium tularensis* from sputum of atypical case of, 903
- in cats, 911
- in chicks, 912
- in dogs, natural occurrence of, used as source of canine distemper virus, 906
- in laboratory animals, 913
- in mice, 912
- T-wave inversion, transient, following paroxysmal tachycardia, 598
- Typing, "reverse", as aid in diagnosis of pneumonia, 1293
- serum, 763
- Ulcerative colitis, treatment of, with phthalylsulfathiazole, 797
- Urea and sulfonamide by mouth, treatment for sulfonamide-resistant gonorrhea, 889
- Urease solution, concentrated, more stable, preparation of, 337
- Uremia, cardiotoxic substances in blood and heart muscle in, 715
- Urinary extracts and urine from rheumatoid patients, effect of subcutaneous injection of, into rats, 19
- Urine, ammonia in, determination of, 447
- and urinary extracts from rheumatoid patients, effect of subcutaneous injection of, into rats, 19
- recovery of penicillin from, 769
- V
- Vascular responses in extremities of man in health and disease, 782 (B. Rev.)
- Vehicle for intravenous administration of fat-soluble hormones, 530
- Venipuncture, effect of distal application of sphygmomanometer cuff on localized venous pressure and aid in, 539
- Venous pressure, localized, effect of distal application of sphygmomanometer cuff on, and aid in venipuncture, 539
- Ventilation recorder for animal experiments, 1205
- Vertebral arteries, aneurysms of; consideration of their etiology, 28
- Vertigo and related conditions, 680
- Vi agglutinative properties for *Bacterium typhosum* demonstrated following infection with malaria parasites, 916
- Viewing device for reading Kahn reactions, 752
- Kahn, with standard illumination for reading tubes in rack, 755
- Virus, influenza, Melbourne strain of, resistance of, to desiccation, 37
- persistence of, on human hand, 121
- Vitamin B complex deficiency, patients with, nutritional macrocytic anemia in, 1226
- C subnutrition, intradermal test for, 464
- D, effects of, on capillary permeability by use of dye T-1824, 48
- W
- War wounds, facial and body prosthesis in relation to, 667
- Waterhouse-Friderichsen syndrome, 703
- associated with meningococcal septicemia, 357
- Weights, organ, of children, nomogram for, 1300
- Wounds, aerobic and anaerobic cultures of, gentian violet-blood agar plates used in, 1090
- healing, role of creatine in cell growth in vitro and its use in, 483
- war, facial and body prosthesis in relation to, 667
- Wright's polychrome methylene blue-eosin stain, 1097
- stain for thick blood films, simple acid water solution for better visualization of malarial parasites using, 1103
- X
- Xenopus laevis daudin (South African clawed frog), reaction of, to human pregnancy serum, 527
- Xylenesulfonic acid, as protein precipitant for sulfonamide determination in blood, 879
- Y
- Yohimbine hydrochloride, 1217
- toxicity of, 1222

